Role of a host-induced arginase of Xanthomonas oryzae pv. oryzae in promoting virulence on rice

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ABSTRACT

The plant bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) causes bacterial blight of rice, which is one of the most destructive diseases prevalent in Asia and parts of Africa. Despite many years of research, how *Xoo* causes bacterial blight of rice is still not completely understood. Here, we show that the loss of the *rocF* gene caused significant decrease in the virulence of *Xoo* in the susceptible rice cultivar IR24. Bioinformatics analysis demonstrated that the *rocF* gene encodes an arginase. Quantitative real-time PCR (qRT-PCR) and Western blot assays revealed that expression of the *rocF* gene was significantly induced by rice and arginine. The *rocF* gene deletion mutant strain showed elevated sensitivity to hydrogen peroxide (H$_2$O$_2$), reduced production of extracellular polysaccharide (EPS) and reduced biofilm formation, all of which are important determinants for the full virulence of *Xoo*, compared to those of 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 *Xoo* on rice because of its contribution to tolerance to reactive oxygen species, production of EPS, and biofilm formation.

**Key words:** *Xanthomonas oryzae* pv. *oryzae*, arginase, virulence, biofilm

INTRODUCTION

The plant bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) causes bacterial blight of rice, which was first characterized in Japan in 1884 and has been one of the most destructive diseases prevalent in Asia and parts of Africa, Australia and Latin (Sere et al. 2005, Nino-Liu et al. 2006). *Xoo* 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 (Gonzalez et al. 2012).

The *Xoo*-rice interaction is an important working model to explain how pathogens inhibit the host plant immune responses (Hilaire et al. 2001). Previous studies indicate that *Xoo* 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 (Buttner et al. 2010, Yu et al. 2016). EPS can enhance the attachment to different environmental surfaces, protect themselves from desiccation and promote the colonization of bacterial pathogens in host by reducing the host responses (Sutherland 1988). Meanwhile, EPS is 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 radical, hydrogen peroxides and high or low pH values (Marquis 1995). The production of EPS and the formation of biofilm are critical for the tolerance to hydrogen peroxide (H$_2$O$_2$) which is an anti-microbial agent to kill *Xoo* (Bae et al. 2018). In addition, the production of EPS and the formation of biofilms in bacteria can be regulated by quorum sensing (QS) (Bodman et al. 1998).

Studies showed that arginine can augment the biofilm formation and polysaccharide intercellular adhesin 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 bacteria (Jakubovics et al. 2015). The arginase pathway is one arm of arginine catabolism in bacterial pathogens which 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 an arginase is responsible for the first step of the arginase pathway which hydrolyzes
arginine to ornithine and urea (Lu 2006). The arginase has been shown to be crucial for its pathogenicity of *Helicobacter pylori* in the human stomach through dysregulating the host immune response, such as inhibiting nitric-oxide (NO) production of macrophages by directly competing with host nitric-oxide synthase, protecting pathogen from damage of reactive oxygen species (ROS), inhibiting human T cell proliferation and reducing the expression of the TCR-zeta chain (CD3 zeta) (Gobert et al. 2001, Zabaleta et al. 2004, Shi et al. 2014, George et al. 2017). In addition, arginase is essential for the survival of *Leishmania donovani* in humans to cause disease (Boitz et al. 2017). This pathway also is 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 the expression of RocF in *Xoo* was induced by host rice and was identified as an arginase. But the reason why it was induced is still unknown. Pathogenic bacteria can activate the transcription of host-induced genes to disrupt host immune response and adapt to highly heterogenous environment in host tissues (Bumann et al. 2007). Recent work has indicated that the expression of genes *acrA* and *dinF* in *Ralstonia solanacearum* can be induced by toxic compounds and are required for the colonization and bacterial wilt virulence in tomato (Brown et al. 2007). Meanwhile, Kachroo found that there’s one rice-induced protein in *Magnaporthe grisea* by using two-dimensional polyacrylamide gel electrophoresis which plays an important role in the process of infection (Kachroo et al. 2010). In addition, studies demonstrated that sRNA genes are related to the adaptation to environmental stress conditions and 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 the virulence of pathogens. Whether the rice-induced arginase in \textit{Xoo} also is important for promoting virulence on rice is still unrevealed.

In this study, we found that the arginase is encoded by the \textit{rocF} gene (Accession NO. WP\_011407409) and is required for the full virulence of \textit{Xoo} on rice. Bioinformatic analysis showed that the \textit{rocF} gene is highly conserved among important plant-pathogenic \textit{Xanthomonas} species. We also obtained the evidence that expression of the \textit{rocF} gene is significantly induced by rice leaf extracts and arginine by using QRT-PCR and Western Blot. Subsequent genetic and phenotypic studies showed that the \textit{rocF} gene plays an important role in the multiple virulence-related functions of \textit{Xoo}, including extracellular polysaccharide (EPS) production, biofilm formation and the tolerance to hydrogen peroxide (\textit{H}_{2}\textit{O}_{2}). To the best of 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 the 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. \textit{Escherichia coli} was grown in Luria-Bertani (LB) medium at 37 °C and the \textit{Xoo} wild-type strain PXO99A and derived mutant strains were cultured in nutrient broth (NB) medium at 28 °C (Qian et al. 2013). The minimal medium XVM1 (20 mM NaCl, 0.5 mM phosphate, 10 mM sucrose, 2 mg/L methionine) containing macerated rice, wheat or tobacco leaves: 20 g of four- to five-week-old leaves of rice, wheat or tobacco were macerated in the liquid nitrogen which were used as leaf extract, then were added to 400 mL XVM1 medium and autoclaved to analyze the expression of the \textit{rocF} gene (Tsuge et al. 2002, Gonzalez et al. 2013).
Antibiotics of kanamycin (50 µg/mL), spectinomycin (25 µg/mL), and gentamicin (25 µg/mL) were added to growth medium as appropriate for selection. Plasmids were transformed into *E. coli* by heat shock and into the *Xoo* wild-type strain PXO99A by electroporation.

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

**Construction of the rocF gene deletion mutant and complementary strains.** The *Xoo* wild-type strain PXO99A was used as the parental strain to obtain the in-frame deletion mutant via the method of allelic homologous recombination which has been previously described (Qian et al. 2013). For complementation of *rocF*, a 1890 bp DNA fragment containing the *rocF* gene and its predicted promoter region was cloned into pUFR047 plasmid. The resulting constructs were transformed into the ΔrocF strain by electroporation to obtain the complementary strain. The PCR primers used in this study are listed in Supplemental Table S1.

**Construction of pSS122 promoter-probe plasmid and β-glucuronidase reporter assay.** To determine the activity of the *rocF* gene promoter, the promoter of the *rocF* gene was predicted by using the online software Promoter 2.0 Prediction Server (http://www.fruitfly.org/seq_tools/promoter.html) and one 376 bp promoter was amplified by PCR using the primers P-rocF-F and P-rocF-R. The amplified promoter was digested with the restriction endonucleases and was then integrated into the pSS122 promoter-probe vector plasmid (which was constructed from pUFR047) by T4 DNA ligase. Then the recombined plasmid was transformed into the wild-type strain by electroporation and grown on NA (NB agar) plates with
gentamicin and ampicillin. In addition, the β- glucuronidase activity assay was performed as described (Pandey et al. 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 the XVM1 medium plates containing 38 µM/mL X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) and different concentrations of arginine at 28 °C for 5-7 days. Meanwhile, 4 to 5-week-old leaves from the susceptible rice cultivar IR24 were inoculated with the wild-type strain which contains the pSS122 plasmid with or without the rocF promoter produced by using the leaf-clipping method, then the leaves of the 7th and 14th days were dipped into the GUS assay buffer containing 38 µM/mL X-Gluc at 37 °C for 3 days. All the leaves were subsequently dipped into ethanol for approximately 5 minutes, which was repeated three times until the chlorophyll disappeared in the rice leaves. Leaves which were inoculated with ddH2O and the wild-type strain as a negative control. At least 20 leaves were inoculated for each treatment and every treatment was repeated three times.

**RNA extraction and quantitative real-time PCR (qRT-PCR) analysis.** The transcriptional level of genes in the PXO99A strains which were cultured in NB liquid medium with or without H2O2 and XVM1 liquid medium with or without rice, wheat, tobacco leaf extracts or 0.1 mM amino acids were analyzed as described with some modifications (Song et al. 2017). Total RNA was extracted with the Trizol reagent (Takara, Dalian, China) according to the manufacture’s protocol and cDNA was synthesized from total RNA using the TransScript All-in-One First-Strand cDNA Synthesis SuperMix (with One-Step gDNA Removal) Kit (TransGen Biotech, Beijing, China) according to the manufacturer’s instructions. QRT-PCR was performed in Applied Biosystem’s QuantStudio™ 6 and 7 Flex Real-Time PCR System (Applied Biosystem’s, Foster City, CA, USA). 100 ng cDNA of each sample was used in qRT-PCR. The relative expression ratio was
calculated by using -ΔΔCt method. The 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 the expression of the RocF protein.** To test the expression level of the RocF protein under different conditions, one integrated PXO99A 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 the rocF gene and this fragment was amplified by PCR. Then the fragment was cloned into the pUFR047 plasmid and the integrated plasmid was transformed into the Xoo wild-type strain PXO99A to obtain a strain in which the region containing the rocF gene was replaced by the FLAG tagged fused fragment. The integrated Xoo wild-type strain PXO99A were cultured in 50 mL 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 6000 rpm for 5 minutes, then the supernatants were discarded and the cell pellets were suspended in 50 mL XVM1 liquid medium with or without rice, wheat, tobacco leaf extracts or 0.1 mM arginine at 28 °C, while shaking at 220 rpm for another 16 h incubation. Bacterial cells were harvested at 10000 rpm, at 4 °C for 10 minutes, and 1 mL RIPA (Radio Immunoprecipitation Assay) lysis buffer with 3 µL PMSF (Phenylmethanesulfonyl fluoride) and 3 µL protease inhibitor cocktail were added to extract the total proteins. The samples were subsequently centrifuged at 10000 rpm, at 4 °C for 5 minutes and transferred the supernatants to new 2 mL tubes containing protein loading buffer before being frozen at -80 °C. 6 µg total proteins of each sample were used in Western blot. Soluble proteins were separated on SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes by using a semidry blot machine (Bio-Rad, USA). Membranes were blocked with 1 × PBST buffer
with 5% nonfat milk and were probed with anti-Flag mouse antibody (1:5000) before being incubated with HRP-conjugated Goat anti-mouse secondary antibody (1:10000). 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 dark at 25 °C (Wang et al. 2018). Then the four- to five-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 method of leaf-clipping described previously (Kauffman, 1973). Lesion lengths were measured 15 days after inoculation. For the bacterial population assay, 3 cm clipped leaf sections were centered on the inoculation sites that were inoculated by PXO99\(^{A}\) strains and ground in 2 mL tube with 1 mL sterile distilled water, and at least 10 leaves were collected for each sample. Bacterial populations were measured by dilution plating of 100 µL 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 has been described previously with some modifications (Li et al. 2012). Briefly, the PXO99\(^{A}\) strains were cultured in NB liquid medium to an optical density of 1.0 at 600 nm. 40 µL bacterial suspensions were transferred to sterilized polystyrene tubes with 4 mL NB liquid medium and incubated at 28 °C, while shaking at 220 rpm overnight, and all 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. The biofilm formed on tubes was visualized by staining with 0.1% crystal violet, after 20 minutes, 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,
v:v) and was measured the absorbance value was measured at an optical density of 590 nm through the use of an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Inc., Santa Clara, CA, USA). The experiment was repeated three times.

The confocal laser scanning microscope (CLSM) technology used to assay the formation of biofilm has been described previously (Tondo et al. 2016). In brief, we transformed the pUFZ75 plasmid, which can express the green fluorescent protein (GFP), into the PXO99A strains. The GFP-labeled PXO99A strains were cultured in NB liquid medium with 10 mM kanamycin to an optical density of 1.0 at 600 nm, 40 µL suspensions were transferred into 4 mL NB liquid medium and then 200 µL mixed suspensions were transferred into sterilized flow-chambers covered with glass slides (cat. no. 155411, Lab-Tek, NUNC, Naperville, IL, USA). All of the flow-chambers were kept in a humidified polyvinylchloride (PVC)-box at 28 °C for 2-3 days without shaking. Biofilm construction was visualized by CLSM (Leica Microsystems Inc., Buffalo Grove, IL, USA) 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 PXO99A strains were cultured in NB liquid medium were grown to an optical density of 1.0 at 600 nm. Then 80 µL bacterial suspensions of each strain were transferred into new sterilized glass bottles with 8 mL NB liquid medium containing 0, 0.1 and 0.2 mM H₂O₂. Meanwhile, bacterial suspensions of the PXO99A strains at an optical density of 1.0 at 600 nm were diluted 5 folds (5×) and 25 folds (25×) and 3 µL diluted bacterial suspensions were dropped onto the surface of NB solid plates with 0, 0.1 and 0.2 mM 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 2-3 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 Extracellular Polysaccharide production.** The PXO99^A strains were cultured on NB agar plates at 28 °C for 2-3 days. Then, cells were incubated in NB liquid medium at 220 rpm, at 28 °C to an optical density of 1.0 at 600 nm. 500 µL bacterial suspensions of the relative strains were transferred to 50 mL sterilized NB liquid medium and incubated at 28 °C with constant shaking at 220 rpm for 5 days. 50 mL bacterial suspension was centrifuged at 10,000 rpm for 10 minutes, the supernatant was transformed into new containers and 100 mL 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 by using SPSS 14.0 (SPSS Inc., Chicago, IL, USA). Significant differences in virulence, bacterial population, EPS production, biofilm formation and gene expressions among different strains were determined via the hypothesis test of percentages (t-test).

**RESULTS**

The RocF protein is highly conserved among many important plant-pathogenic *Xanthomonas* species. To better understand the *rocF* gene, bioinformatics analysis was performed. The open reading frame of the *rocF* gene (locus tag: PXO_RS22625) is 924 bp and the nucleotide position in the genome is from 4951804 to 4952727 (Fig. 1A). The RocF protein contains an arginase domain (from residues 6 to 300) which was predicted by using SMART
(http://smart.embl-heidelberg.de/) and the molecular weight is 3.316 kDa as predicted by using the ExPASy ProtParam tool (https://web.expasy.org/protparam/). Amino acid sequence alignment analysis demonstrated that the RocF protein is highly conserved among the important plant-pathogenic *Xanthomonas* species such as, *Xanthomonas oryzae* pv. *oryzae* KACC 10331 (Accession: AAW73689), *Xanthomonas campestris* pv. *campestris* str. B100 (Accession: WP_012439504), *Xanthomonas campestris* pv. *campestris* str. ATCC 33913 (Accession: NP_639266), *Xanthomonas oryzae* pv. *oryzicola* BLS256 (Accession: AEQ98376), *Xanthomonas campestris* pv. *campestris* str. 8004 (Accession: AAY51054), *Xanthomonas gardneri* (Accession: WP_074058724) and *Xanthomonas oryzae* pv. *oryzae* PXO86 (Accession: AJQ85091) (Fig. 1B).

**The expression of the rocF gene is induced by rice leaf extracts and arginine in vitro.** To determine whether the expression of the *rocF* gene can be induced by rice leaf extracts, we performed qRT-PCR experiments to determine the transcriptional level of the *rocF* gene in XVM1 liquid medium with or without rice leaf extracts as described in the Experimental Procedures. The results of the qRT-PCR showed that the transcriptional level of the *rocF* gene is approximately 4 folds higher in XVM1 medium in the presence of rice leaf extracts than in XVM1 medium, the transcriptional level of the *rocF* gene also can be significantly induced by non-host plant leaf extracts, including wheat and tobacco. The same method was used to detect the transcriptional level of the *rocF* gene in XVM1 liquid medium with 0.1 mM arginine. The results of the qRT-PCR experiment showed that the transcriptional level of the *rocF* gene is approximately 5 folds higher in the XVM1 medium in the presence of 0.1 mM arginine than in XVM1 medium in the absence of arginine (Fig. 2A). We also measured the transcriptional level of the *rocF* gene in the XVM1 medium containing seven different amino acids at a concentration of 0.1 mM. The results showed that the expression of the *rocF* gene was significantly induced by glutamate and ornithine which were metabolites from arginine, but not histidine, methionine, proline, phenylalanine and
leucine (Supplemental Fig. S1). All these results indicate that the expression of the rocF gene can be significantly induced by rice leaf extracts and arginine.

Since the expression of the rocF gene can be significantly induced by rice leaf extracts and arginine at transcriptional level, we then investigated whether the expression of the RocF protein can be induced at the same conditions by using western blot. The integrated PXO99A strain was constructed in which the 5’ terminus of the rocF gene was labeled with a FLAG tag as described in the Experimental Procedures. The PXO99A strain with FLAG-tagged rocF gene was cultured in 50 mL XVM1 liquid medium with or without rice leaf extracts or 0.1 mM arginine, and the total proteins were extracted and analyzed as described in the Experimental Procedures. Western blotting analysis showed that the presence of rice leaf extracts or arginine resulted in a significant increase in the expression of the RocF protein 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. Meanwhile, the expression of the RocF protein also can be significantly induced by wheat and tobacco leaf extracts. Under the same test conditions, the expression of the α subunit of RNA polymerase was analyzed as an internal control (Fig. 2B). These data indicate that the expression of the rocF gene and RocF can be induced by host and non-host leaf extracts and arginine maybe the potential inducer.

The promoter activity of the rocF gene is induced by arginine during pathogen infection.

To further confirm that the promoter of the rocF gene was also functional in rice leaf, the the β-glucuronidase activities of the PXO99A strains with or without the pSS122 plasmid containing the promoter of the rocF gene in rice leaves were determined as described in the Experimental Procedures. Few indigo-blue staining was observed in major longitudinal veins of rice leaves after 7 days inoculation of the PXO99A strain with the pSS122 plasmid containing the promoter of the
rocF gene, but no staining was observed in major longitudinal veins of rice leaves which were
infected by the PXO99A strains with or without the pSS122 reporter plasmid. Staining intensity
increased during the development of rice. Intense indigo-blue staining was also observed in major
longitudinal veins of rice leaves after 14 days inoculation of the PXO99A strain with the pSS122
plasmid containing the promoter of the rocF gene, but indigo-blue staining still can’t be observed
in major longitudinal veins of rice leaves (Fig. 3A). These results demonstrate that the activity of
the rocF promoter maybe increased by components of the rice leaves during the infection of the
PXO99A strain.

The results of qRT-PCR and Western blot showed that the expression of rocF was significantly
induced by arginine, therefore, we speculated that the arginine in rice leaves is the component to
increase the activity of the rocF gene. Therefore, we determined the activity of the rocF gene on
XVM1 medium plates with or without 0.1 mM arginine. Indigo staining was observed of the
PXO99A strain with the pSS122 plasmid containing the promoter of the rocF gene, while no
staining was observed of the PXO99A strain with or without the pSS122 plasmid (Fig. 3B). All the
results suggest that the activity of the promoter of the rocF gene is significantly induced by
arginine during pathogen infection.

_the rocF gene is required for the full virulence of X. oryzae pv. oryzae._ To investigate the
potential biological functions of the rocF gene in PXO99A, a rocF gene deletion mutant strain
(ΔrocF) and a complementary strain [ΔrocF (rocF)] were constructed. The qRT-PCR results
showed that the corresponding region of the rocF gene was deleted successfully (Supplemental
Fig. S2). The virulence assay for PXO99A strains in the susceptible rice cultivar IR24 was
performed by the leaf-clipping method (Guo et al., 2015). The lesion lengths were measured 15
days post-inoculation (Fig. 4A) and the data were presented in (Fig. 4B). The results showed that
the wild-type strain caused a lesion length of 26 ± 0.8 cm while the ΔrocF strain just caused a lesion length of just 12.5 ± 1.0 cm in all infected leaves. These data indicate the virulence of PXO99^A is significantly decreased after the deletion of the rocF gene. The virulence of ΔrocF strain in the leaves of susceptible rice cultivar IR24 was restored in the complementary strain ΔrocF(rocF), which caused a lesion length of 23 ± 1.2 cm. Meanwhile, we assayed the colonization of PXO99^A on rice leaves at 0, 8 and 15 day post-inoculation. The growth of the ΔrocF strain was significantly reduced on rice leaves compared with the growth of the wild-type strain and the reduced bacterial population was restored to the of wild-type levels by complementation (Fig. 4C). To determine whether the deletion of the rocF gene 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, ΔrocF and ΔrocF(rocF) strains in their ability to grow in NB liquid medium (Supplemental Fig. S3). The results indicate that deletion of the rocF gene 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 gene is required for PXO99^A to achieve full virulence on rice.

Deletion of the rocF gene reduces extracellular polysaccharide (EPS) production by X. oryzae pv. oryzae. The formation of EPS 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 the rocF gene affects the production of EPS in PXO99^A. The wild-type, ΔrocF and ΔrocF(rocF) strains were grown in NB liquid medium for 5 days, and EPS was extracted from the cultures and was quantified as described in the Experimental Procedures. The results showed that EPS production was reduced by approximately 50% in ΔrocF strain compared with the wild-type strain and there was no
significant difference in EPS production between $\Delta rocF(rocF)$ strain and wild-type strain (Fig. 5A). In addition, our qRT-PCR data showed that deletion of the rocF gene caused significantly decreased expression of genes related to polysaccharide biosynthesis process including $PXO_{RS15910}$ ($GumC$), $PXO_{RS14800}$ (alpha-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 the rocF gene plays an important role in EPS production of PXO99A.

Loss of the rocF gene 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 et al. 2007, Vu et al. 2009). Since deletion of the rocF gene reduced the production of EPS, it is likely that the rocF gene is related to the biofilm formation in PXO99A.

To test this hypothesis, we determined biofilm formation in the wild-type, $\Delta rocF$ and $\Delta rocF(rocF)$ strains of PXO99A on the surface of polystyrene tubes. The results showed that biofilm formation on the surface of polystyrene tubes by the $\Delta rocF$ strain was significantly reduced compared to 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) and crystal violet staining of biofilm produced by the complemented strain was restored to level observed for the wild-type strain. These results indicate that the rocF gene contributes to the biofilm formation of PXO99A on the surface of polystyrene tubes. To further validate these results, we used the confocal laser scanning microscope (CLSM) technology to observe the biofilm formation of PXO99A strains as described in the Experimental Procedures. The results showed that the $\Delta rocF$ strain produced a less organized biofilm than the
wild-type strain. Meanwhile, the thickness of the biofilm produced by the ΔrocF strain is approximately 20 to 30 µm, which is much thinner than the approximately 50 to 60 µm thick biofilm produced by the wild-type strain (Fig. 6C). These data indicate that the deletion of the rocF gene negatively influenced the biofilm formation. Therefore, the data from these two assays demonstrate that the rocF gene is required for biofilm formation of PXO99A.

The rocF gene contributes to hydrogen peroxide 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 hydrogen peroxide (H₂O₂) (Vu et al. 2009). Therefore, to investigate the role of the rocF gene in resistance to H₂O₂, we carried out the experiments as described in the Experimental Procedures. The results showed that the growth of the ΔrocF strain was weaker on 0.1 mM and 0.2 mM H₂O₂ plates than the growth of the wild-type strain (Fig. 7A) and the similar result was found in 8 ml NB liquid medium with 0, 0.1 and 0.2 mM H₂O₂ (Fig. 7B). In addition, the growth rate of the ΔrocF strain was significantly inhibited in the presence of 0.2 mM H₂O₂ in NB liquid medium compared with the wild-type and ΔrocF(rocF) strains (Fig. 7C). All the results indicated that the ΔrocF strain was significantly more sensitive to H₂O₂ than the wild-type strain. In addition, the complementary strain could fully rescue their tolerance to H₂O₂. Meanwhile, the genes including PXO_RS17325 (catalase HPII), PXO_RS22555 (catalase), PXO_RS05475 (ahpC), PXO_RS05465 (LysR family transcriptional regulator) and PXO_RS06520 (Fur family transcriptional regulator), which are involved in H₂O₂ detoxification and adaption, were measured. Their transcriptional levels were determined in the wild-type strain and the ΔrocF strain in the presence of 0.1 mM H₂O₂ by qRT-PCR. The results showed that the expression of these genes was significantly reduced in the ΔrocF strain compared to that in the
wild-type strain (Fig. 7D). Taken together, these data demonstrated that the *rocF* gene is indeed involved in the hydrogen peroxide stress tolerance of PXO99A.

**DISCUSSION**

In this study, we identified a novel *Xoo* virulence gene, *rocF*, which encodes an arginase. Mutant of the *rocF* gene showed dramatically reduced virulence on rice. The results of this study indicate that decreased EPS production, reduced Biofilm formation and enhanced sensitivity to H$_2$O$_2$ are major reasons of 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 protect pathogens against toxic compounds from host to enhance pathogenicity, for example, H$_2$O$_2$ (Guo et al. 2015). The *detR* mutant showed reduced virulence of *Xoo* which resulted from a reduction of EPS and intolerance to ROS (MP et al. 2016). The deletion of the *rocF* gene in *Xoo* also resulted in the loss of extracellular polysaccharide (EPS) production, meanwhile, the transcriptional level of *PXO_RS15910* (*gumC*) was decreased in Δ*rocF* compared with wild-type. *GumC* is one of the important *gum* genes which is responsible for transport and polymerization and is required for EPS synthesis, mutant of this gene showed reduced virulence (Kim et al. 2009). Therefore, we conclude that the *rocF* gene 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. *Xoo* 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 the infection and colonization and is important for the tolerance to environment stresses of *Xoo* (Boher et al. 1997,
Crossman et al. 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 the rocF gene is crucial for the formation of biofilm to regulate the virulence of Xoo. Xoo successfully infect rice depending on the ability to counteract H₂O₂, its catalases are essential for the detoxification process of H₂O₂. It has been reported that the OxyR-regulated catalase CatB functions as an important factor to detoxify H₂O₂ in order to promote the bacterial pathogenesis of Xoo on rice (Yu et al. 2016). In our study, ΔrocF was hypersensitive to H₂O₂ and very small bacterial population survived under the condition of 0.2 mM H₂O₂. In addition, we measured the transcriptional levels of catalases in ΔrocF because catalase is required for the detoxification of H₂O₂ in Xanthomonas spp. during their early stages of infection. The results of qRT-PCR indicated that the transcriptional levels of PXO_RS17325 (catalase HPII), PXO_RS22555 (catalase) and PXO_RS05475 (ahpC) were deregulated. These findings lead us to conclude that the rocF mutant caused dramatically reduced virulence of Xoo on rice, maybe due to the deregulation of detoxifying enzymes, such as catalases.

Although EPS production, biofilm formation and tolerance to hydrogen peroxide (H₂O₂) contribute to the virulence of Xoo on rice, more work will be needed to increase our understanding of the underlying molecular mechanisms of their regulation in Xoo. 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 (Ziveri et al. 2017). In our study, we the rocF gene encodes an arginase which is an arginine metabolic enzyme and the transcriptional levels of genes that are related to EPS biosynthesis or detoxification of H₂O₂ were reduced, this leads us to conclude that the metabolic enzyme arginase maybe also can combine the promoter region of these genes to regulate their transcription.
In addition, arginine has been characterized as an important nitrogen source for plants (Slocum 2005). More importantly, arginine is a common substrate used by nitric oxide synthase (NOS) to produce nitric oxide (NO) and NO is a diffusible molecular messenger that plays a key role in the rapid induction of the plant immune response (Vidhyasekaran 2014). Therefore, whether the rocF mutant caused dramatically reduced virulence of \( X_{oo} \) on rice is associated with the metabolic of arginine then inhibit the NO production of rice needs our further studies.

In summary, this study demonstrated that after \( X_{oo} \) infects rice, the arginine in rice is used by \( X_{oo} \) to induce the expression of the rocF gene which contributes to the EPS production, biofilm formation and hydrogen peroxide (\( H_2O_2 \)) detoxification. Notably, all of these functions are major factors, that contribute to the reduced virulence of the rocF gene deletion mutant strain. 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 \( X_{oo} \), but also is helpful to our understanding of the pathogenic mechanisms of \( X_{oo} \). In future studies, it will be interesting to determine how the rocF gene contributes to the expression of important genes in EPS production and \( H_2O_2 \) detoxification and adaption.

ACKNOWLEDGEMENTS

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LITERATURE CITED


TABLES

Table 1. Bacterial strains and plasmids used in this study.

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<th>Strains or plasmids</th>
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<td>DH5a</td>
<td>F-, φ80dlacZΔM15, Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rk-,mk+), phoA, supE44, λ-, thi-1, gyrA96</td>
<td>This study</td>
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<td>PXO99A</td>
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<td>(Qian et al. 2013)</td>
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<td>ΔrocF</td>
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<tr>
<td>PXO99A(pSS122::rocF)</td>
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<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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pK18mobsacB allelic exchange suicide vector, sacB oriT(RP4); Km\(^R\) (Qian et al. 2013)
pK18-rocF pK18mobsacB with two PXO_03177 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-rocF pUFR047 carrying gene rocF with its native promoter region; Gm\(^R\), Amp\(^R\) This study
pUFR047-rocF-Flag pUFR047 carrying gene rocF 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, Inc\(W\), Gm\(^R\), Ap\(^R\) (Gonzalez et al. 2012)
pSS122::rocF pSS122 carrying gene rocF with its native promoter region, Gm\(^R\), Ap\(^R\) This study

\(^a\) Km\(^R\), Gm\(^R\), Amp\(^R\) = kanamycin, gentamicin, ampicillin, respectively.

**FIGURE CAPTIONS**

**Figure 1. Bioinformatics analysis of the rocF gene.** (A) Schematic diagram of the rocF gene in the genome of *X. oryzae* pv. *oryzae* PXO99\(^A\). The open arrows indicate location, length and orientation of the ORFs. The middle element shows the domain structure analysis of the RocF protein which was performed by using SMART software. The lower element shows that the rocF gene was amplified by PCR primers P1 and P2 and was cloned into the pUFR047 plasmid for complementation of the rocF mutant. (B) The identity of arginase homologues in important plant-pathogenic *Xanthomonas* species. \(^a\) *Xoo* PXO99\(^A\): *X. oryzae* pv. *oryzae* PXO99\(^A\), *Xoo* PXO86: *X. oryzae* pv. *oryzae* PXO86, *Xoo* KACC10331: *X. oryzae* pv. *oryzae* KACC10331, *Xoc* BLS256: *X. oryzae* pv. *oryzicola* BLS256, *Xcc* B100: *X. campestris* pv. *campestris* B100, *Xcc* ATCC33913:
X. campestris pv. campestris str ATCC33913, Xcc 8004: X. campestris pv. campestris str. 8004. "
Domains were predicted by the Smart website http://smart.embl-heidelberg.de/. According to a BLASTP search.

Figure 2. The expression of the rocF gene in different media. (A) The mRNA expression levels of the rocF gene in different XVM1 medium were detected by using qRT-PCR. The relative transcriptional levels of these genes were calculated by qRT-PCR with respect to the transcriptional levels of the corresponding transcriptional levels in the wild-type and the 16S rRNA as the internal standard. (B) Analysis of the protein expression level of the RocF protein in different XVM1 medium by using Western blot. 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. XVM1: negative control, XVM1#: XVM1 medium with 0.1 mM/L arginine, XVM1###: XVM1 medium with 5 g/L rice leaf extracts, XVM1####: XVM1 medium with 5 g/L wheat leaf extracts, XVM1#####: XVM1 medium with 5 g/L tobacco leaf extracts. Values are the means ± SDs from three independent experiments. Asterisks above error bars indicate significant differences compared with XVM1 medium (t-test, **P< 0.01).

Figure 3. GUS activity determination of the promoter of the rocF gene in rice leaves and under arginine condition. (A) GUS activity determination of the PXO99A strains with or without the pSS122 plasmid containing the promoter of the rocF gene infected rice leaves stained by GUS assay buffer containing X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid). The leaves were acquired after the inoculation of 7 and 14 days. The wild-type strain without the pSS122 vector and ddH₂O infected leaves were used as negative control. The indigo-blue staining indicates increased GUS activity of the promoter of the rocF gene. DPI: days post-inoculation. (B) GUS
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**Figure 4. The results of the pathogenicity test of X. oryzae pv. oryzae on host rice.** (A) Disease symptoms caused by the wild-type, ΔrocF and ΔrocF(rocF) strains on four- to five-week-old susceptible rice cultivar IR24 leaves. Representative pictures were taken 15 days post inoculation. Sterilized water inoculated rice leaves as a negative control. (B) Lesion length caused by the wild-type, ΔrocF and ΔrocF(rocF) strains (OD₆₀₀ = 0.5) on four- to five-week-old susceptible rice cultivar IR24 leaves by using leaf clipping method. The lesion lengths were calculated 15 days post inoculation. (C) Bacterial populations of the wild-type, ΔrocF and ΔrocF(rocF) strains (OD₆₀₀ = 0.5) on four- to five-week-old susceptible rice cultivar IR24 leaves by using 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 NB solid plates to count the number of bacterial populations (CFU). The bacterial populations were presented as the lg CFU per leaf. Values are the means ± SDs from three independent experiments (n = 50). The asterisks above the error bars indicate significant differences compared with the wild-type strain (t-test, **P< 0.01).

**Figure 5. Effects of rocF gene deletion on the production of extracellular polysaccharide (EPS) by X. oryzae pv. oryzae.** (A) Determination of EPS production by the wild-type, ΔrocF and ΔrocF(rocF) strains grown in 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) Determination
of the transcriptional levels of seven sugar transport and biosynthesis genes in the wild-type and ΔrocF strains grown in NB liquid medium by using qRT-PCR. The relative transcriptional levels of these genes were calculated by qRT-PCR with respect to the transcriptional levels of the corresponding transcriptional levels in the wild-type and the 16S rRNA as the internal standard. Values are the means ± SDs from three independent experiments. The asterisks above error bars indicate significant differences compared with the wild-type strain (t-test, **P< 0.01).

**Figure 6. Effects of rocF gene deletion on the formation of biofilm by X. oryzae pv. oryzae.**

(A) Biofilm formation by the wild-type, ΔrocF and ΔrocF(rocF) strains on polystyrene tubes stained with crystal violet. (B) Biofilm production quantification of the wild-type, ΔrocF and ΔrocF(rocF) strains on polystyrene tubes corresponding to part A. The value of OD_{590} was measured after the stained tubes were incubated with ethanol-acetone. (C) Confocal laser scanning microscopy was performed for observing 3-dimension structure of biofilm formation by the wild-type and ΔrocF strains. Images were obtained by using a 20 × objective. Values are the means ± SDs from three independent experiments. The asterisks above the error bars indicate significant differences compared with the wild-type (t-test, **P< 0.01).

**Figure 7. The observation of the resistance to hydrogen peroxide (H_2O_2) of X. oryzae pv. oryzae.** (A) The wild-type, ΔrocF and ΔrocF(rocF) strains were pre-cultured to the exponential phase of the indicated value (OD_{600} = 1.0) in NB liquid medium which as the initial cultures (1 X), then was diluted 5 folds (5 X) and 25 folds (25 X). Three microliters 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 H_2O_2 to observe their growth defects. (B) Eight microliters of the initial cultures in part A were transformed into eight milliliter NB liquid mediums with 0, 0.1 and 0.2 mM H_2O_2 to observe their growth defects, pictures were taken of each sample at 0, 24 and 48 h. (C) Twenty microliters of
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SUPPLEMENTARY MATERIALS

Role of a host-induced arginase of Xanthomonas oryzae pv. oryzae in promoting virulence on rice

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†The first two authors contribute equally to this research.
Supplemental Fig. S1. The expression of the rocF gene in different medium. The mRNA expression levels of the rocF gene in different XVM1 mediums were detected by using qRT-PCR. The relative transcriptional levels of these genes were calculated by qRT-PCR with respect to the transcriptional levels of the corresponding transcriptional levels in the wild-type and the 16S rRNA as the internal standard. 1: XVM1 medium as negative control, 2: XVM1 medium with 0.1 mM methionine, 3: XVM1 medium with 0.1 mM proline, 4: XVM1 medium with 0.1 mM phenylalanine, 5: XVM1 medium with 0.1 mM leucine, 6: XVM1 medium with 0.1 mM histidine, 7: XVM1 medium with 0.1 mM ornithine, 8: XVM1 medium with 0.1 mM glutamate. The relative transcriptional levels of these genes were calculated by qRT-PCR with respect to the transcriptional levels of the corresponding transcriptional levels in the wild-type and the 16S rRNA as the internal standard. Values are the means ± SDs from three independent experiments. Asterisks above error bars indicate significant differences compared with XVM1 medium (t-test, **P< 0.01).
Supplemental Fig. S2. The construction of the *rocF* gene mutant strain of *X. oryzae pv. oryzae* PXO99A. (A) The *rocF* gene mutant of the wild-type strain was tested by PCR. (B) The *rocF* mutant strains were confirmed by using special primers from the *rocF* gene through PCR. (C) The expression of the *rocF* gene in the wild-type, Δ*rocF* and Δ*rocF*(rocF) strains was determined by qRT-PCR. The relative transcriptional levels of these genes were calculated by qRT-PCR with respect to the transcriptional levels of the corresponding transcriptional levels in the wild-type and the 16S rRNA as the internal standard. Three replicates were used for each treatment and the experiment was repeated three times. Values are the means ± SDs from three independent experiments. Asterisks above error bars indicate significant differences compared with the wild-type (t-test, **P< 0.01).
Supplemental Fig. S3. The growth rates of *X. oryzae* pv. *oryzae* PXO99A strains.

The wild-type, \( \Delta rocF \) and \( \Delta rocF(rocF) \) strains were pre-cultured to the exponential phase of the indicated value (\( \text{OD}_{600} = 1.0 \)) in NB liquid medium then two hundred microliters of the cultures were transformed into twenty milliliter NB liquid mediums to observe their growth rates, the values of each samples at \( \text{OD}_{600} \) every two hours. Three replicates were used for each treatment and the experiment was repeated three times. ● Represented the wild-type strain (PXO99A), ♦ Represented the \( rocF \) mutant strain (\( \Delta rocF \)), ▲ Represented the complementary strain of \( \Delta rocF \) (\( \Delta rocF(rocF) \)).

Values are the means ± SDs from three independent experiments. Asterisks above error bars indicate significant differences compared with the wild-type (t-test, **P< 0.01).

SUPPLEMENTAL TABLE S1. Primers used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)a</th>
<th>Purpose/Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>For mutant construction</td>
<td></td>
<td></td>
<td></td>
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</table>

\( a \) Sequences of primers in the forward direction are shown.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>rocF-1F</td>
<td>CGGAATTCCGCCACAGCAGCCTTGACGACC</td>
<td>To amplify a 404 bp upstream homologue arm of gene rocF</td>
</tr>
<tr>
<td>rocF-1R</td>
<td>GAACGCCAATCAGGGAAACC</td>
<td>This study</td>
</tr>
<tr>
<td>rocF-2F</td>
<td>GGTTCCTGGATTGGCGTTCGACGCTGATGCGGGATTG</td>
<td>To amplify a 710 bp downstream homologue arm of gene rocF</td>
</tr>
<tr>
<td>rocF-2R</td>
<td>GCTCTAGAGCCGCTTGATACGGCCTC</td>
<td>This study</td>
</tr>
<tr>
<td>v-rocF-F</td>
<td>GTTTCCCTGTATGGCGTGTTCC</td>
<td>To amplify 568 bp fragment of gene rocF for mutant confirmation</td>
</tr>
<tr>
<td>v-rocF-R</td>
<td>CGATCGGCTGCTGCTTGAT</td>
<td>For Complementation</td>
</tr>
</tbody>
</table>

**For Complementation**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CΔrocF-F</td>
<td>CGGAATTCCGCCACAGCAGCCTTGACGGGCAAAAACAGCGCTTCGCATGAAAGACGTTGCGG</td>
<td>To amplify a 1890 bp fragment containing gene rocF and its native promoter</td>
</tr>
<tr>
<td>CΔrocF-R</td>
<td>GGGGTACCCCACTCCTCATGGGATAAAAGCGGCGCGGTTGCCGCAAATCGAACAAATTCAGG</td>
<td>This study</td>
</tr>
</tbody>
</table>

**For C-terminal Flag tag fusion in genome level**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>rocF-Flag-1F</td>
<td>CGGGATCCCCCGCCACACTGCCGACom</td>
<td>To amplify a 438 bp upstream homologue arm of gene rocF-Flag</td>
</tr>
<tr>
<td>rocF-Flag-1R</td>
<td>CGGGATGACTACAAAGACGACGACGA</td>
<td>This study</td>
</tr>
<tr>
<td>rocF-Flag-2F</td>
<td>GATCATTGTGCGTCTGTCTTGAATTGCT</td>
<td>To amplify a 337 bp downstream homologue arm of gene rocF-Flag</td>
</tr>
<tr>
<td>rocF-Flag-2R</td>
<td>GCTCTAGACCGGAAAGCCGCTG</td>
<td>This study</td>
</tr>
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</table>

**For GUS assay**

<table>
<thead>
<tr>
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<th>Sequence</th>
<th>Function</th>
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</thead>
<tbody>
<tr>
<td>P-rocF-F</td>
<td>CGGGGTACCAACCGCAAATCCGGGAAACCC</td>
<td>To amplify a fragment containing 376 bp gene rocF promoter</td>
</tr>
<tr>
<td>P-rocF-R</td>
<td>CCCAAGCTTTCAGGCTTACGCGATGGAATAA</td>
<td>This study</td>
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**For qRT-PCR (quantitative real-time PCR)**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA-F</td>
<td>TTCATGGAGTCTGCTTTCAGCAGG</td>
<td>The internal control for relative quantification</td>
</tr>
<tr>
<td>16S rRNA-R</td>
<td>GTCAAGTCATCGGCTTGG</td>
<td>This study</td>
</tr>
<tr>
<td>qRT-rocF-F</td>
<td>TCATTGATCGACAGATCCGA</td>
<td>To determine the transcriptional level of the gene rocF</td>
</tr>
<tr>
<td>qRT-rocF-R</td>
<td>GCACGTCCTCGACGATATTT</td>
<td>This study</td>
</tr>
<tr>
<td>qRT- PXO_RS08055-F</td>
<td>ACCTGTGGAATGCGCAATG</td>
<td>To determine the transcriptional level of the gene PXO_RS08055</td>
</tr>
<tr>
<td>qRT-</td>
<td>CAGATCGCACCAGTACAAGC</td>
<td>This study</td>
</tr>
<tr>
<td>Gene ID</td>
<td>Forward Primer</td>
<td>Reverse Primer</td>
</tr>
<tr>
<td>-------------</td>
<td>------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>PXO_RS08055</td>
<td>CAAGGTCTACGAAAACGGCC</td>
<td>To determine the transcriptional level of the gene PXO_RS10635</td>
</tr>
<tr>
<td>PXO_RS08055</td>
<td>CTGCCTGCACGTAGTCTCTC</td>
<td>This study</td>
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<tr>
<td>PXO_RS10635-R</td>
<td>GCTGATCGAGGAAAAGAGCG</td>
<td>This study</td>
</tr>
<tr>
<td>PXO_RS14055-R</td>
<td>AAAGATCGCCAACACGTACG</td>
<td>This study</td>
</tr>
<tr>
<td>PXO_RS11405-R</td>
<td>CCAGTGTCATGTTGTCGTGG</td>
<td>This study</td>
</tr>
<tr>
<td>PXO_RS11410-R</td>
<td>GCTGATCGAGGAAAAGAGCG</td>
<td>This study</td>
</tr>
<tr>
<td>PXO_RS14800-R</td>
<td>AAAGAAGGTTTGTCGTCGCC</td>
<td>This study</td>
</tr>
<tr>
<td>PXO_RS15910-R</td>
<td>AAAGAAGGTTTGTCGTCGCC</td>
<td>This study</td>
</tr>
<tr>
<td>PXO_RS17325-F</td>
<td>ACTTTGCTGCCTCCGGATAT</td>
<td>To determine the transcriptional level of the gene PXO_RS17325</td>
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<tr>
<td>PXO_RS17325-R</td>
<td>GCACCAACTCTCCTCGGGAATG</td>
<td>This study</td>
</tr>
<tr>
<td>PXO_RS05465-R</td>
<td>CGACAACTATGCTGCTGG</td>
<td>To determine the transcriptional level of the gene PXO_RS05465</td>
</tr>
<tr>
<td>PXO_RS05465-R</td>
<td>CACAAGCTGATCGGATCTCAC</td>
<td>To determine the transcriptional level of the gene PXO_RS05465</td>
</tr>
<tr>
<td>PXO_RS05465-R</td>
<td>GTAATGATGATGGAAGTGGCC</td>
<td>To determine the transcriptional level of the gene PXO_RS05465</td>
</tr>
<tr>
<td>PXO_RS05465-R</td>
<td>ATTAAGTCATCGTCCCTCC</td>
<td>To determine the transcriptional level of the gene PXO_RS05465</td>
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<tr>
<td>PXO_RS05465-R</td>
<td>ACGCGATGAACAGTGTCCC</td>
<td>To determine the transcriptional level of the gene PXO_RS05465</td>
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<td>PXO_RS05465-R</td>
<td>ACTTTGCTGCCTCCGGATAT</td>
<td>To determine the transcriptional level of the gene PXO_RS05465</td>
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<td>PXO_RS05465-R</td>
<td>ACTTTGCTGCCTCCGGATAT</td>
<td>To determine the transcriptional level of the gene PXO_RS05465</td>
</tr>
</tbody>
</table>
METHODS USED IN THIS SECTION

Bacterial growth assays in NB liquid medium

To investigate whether the deletion of the rocF gene influences the normal growth rate of *X. oryzae* pv. *oryzae* PXO99A, we cultured the XOO strains in NB liquid medium to an optical density of 1.0 at 600 nm and transferred 200 µL bacterial suspensions to new sterilized bottles containing 20 mL NB liquid medium. The values for optical density at 600 nm were recorded every 2 h for 32 h and pictures were obtained every 24 h.