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Characterization of *Burkholderia glumae* Putative Virulence Factor 11 (PVF11) via Yeast Two-Hybrid Interaction and Phenotypic Analysis

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In this study, *PVF11* was selected among 20 candidate pathogenesis-related genes in *Burkholderia glumae* based on its effect on virulence to rice. PVF11 was found to interact with several plant defense-related WRKY proteins as evidenced through yeast-two hybrid analysis (Y2H). Moreover, PVF11 showed interactions with abiotic and biotic stress response-related rice proteins, as shown by genome-wide Y2H screening employing PVF11 and a cDNA library from *B. glumae*-infected rice. To confirm the effect of PVF11 on *B. glumae* virulence, *in planta* assays were conducted at different stages of rice growth. As a result, a PVF11-defective mutant showed reduced virulence in rice seedlings and stems but not in rice panicles, indicating that PVF11 involvement in *B. glumae* virulence in rice is stage-dependent.

Keywords **:** *Burkholderia glumae*, virulence, yeast two hybrid analysis

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Burkholderia glumae is a Gram-negative bacterium causing panicle blight as well as seedling and grain rot in rice. The disease was first reported in Japan in 1956, subsequently spreading to many other countries and is currently considered as a major threat to rice cultivations worldwide (Cui et al., 2016; Goto and Ohata, 1956; Ham et al., 2011). The yield loss caused by *B. glumae* can reach 75% in heavily infested fields (Trung et al., 1993). *B. glumae*, as well as other members in the *Burkholderia* genus, is characterized by a high genomic flexibility enabling it to develop various adaptation strategies and virulence factors to overcome stress conditions in the environment and inside the host (Mannaa et al., 2019; Seo et al., 2015). Moreover, *B. glumae* can use additional strategies to efficiently induce severe disease. For example, *B. glumae* cooperatively interacts with the pathogenic fungus, *Fusarium graminearum*, promoting disease progression in rice (Jung et al., 2018).

The pathogenicity of *B. glumae* has been extensively studied and some virulence factors have been identified (Cui et al., 2016). Yet, additional virulence factors could participate in disease-causing mechanisms. Studying the manner by which pathogens interact with host defense machineries is crucial to understand the strategies allowing them to overcome or suppress host defense, resulting in enhanced virulence (Mukhtar et al., 2011).

The yeast two-hybrid (Y2H) system, developed in 1989, is a powerful genetic tool to investigate protein-protein interactions (Fields and Song, 1989). The system has been subjected to modifications and improvements to enhance its efficiency and broaden its application. The Y2H system is widely used to explore the interactions of a wide range of proteins from yeast, bacteria, animals, and plant systems (Lin and Lai, 2017). Y2H assays were employed in this study to investigate the interactions between candidate *B.*

glumae effectors with rice defense-related proteins.

A previous transcriptome analysis conducted on *B. glumae* under *in planta* and *in vitro* conditions resulted in the selection of candidate genes related to bacterial virulence that were highly expressed in *B. glumae* obtained from infected rice tissues (Kim et al., 2014). We have selected 20 of these genes for further investigations. The objective of this study was to identify, among the proteins encoded by these genes, factors interacting with rice defense-related proteins and investigate their role in virulence.

To this end, a preliminary assay was performed at the vegetative stage using mutant strains defective in each of the 20 genes in question. The 20 putative virulence factor (PVF) mutants were generated using standard general DNA manipulation techniques (Sambrook et al., 1989). Briefly, the bacterial strains, *B. glumae* BGR1 and *Escherichia coli*, were grown at 37°C in LB media and specific antibiotics were used according to the purpose. The internal regions of target genes were amplified using primers flanked by restriction enzyme sites. The amplified internal regions of target genes and pVIK112 suicide vector were digested using the same restriction enzymes and ligated by T4 DNA ligase (Kalogeraki and Winans, 1997). Competent *E. coli* DH5α λ*pir* cells were transformed with the recombinant vectors. These were then transformed into BGR1 using *E. coli* S17-1 *λpir*. The complemented mutants were generated by amplification of the complete open reading frame (ORF) of the target gene and cloning into the pRK415 expression vector (Keen et al., 1988). The constructed expression vectors were then transformed into the respective defective mutant strains. All bacterial strains and primers used are listed in Supplementary Tables 1 and 2.

To test the interaction between PVF11 and rice plant defense-related WRKY proteins, Y2H analysis was performed using *Saccharomyces cerevisiae* strains, AH109 and Y187. Strain AH109 expresses *HIS3*, *ADE2*, *MEL1*, and *lacZ* reporter genes under the control of three thoroughly heterologous GAL4 transcriptional responsive upstream activation sequences (UAS) and TATA promoter elements (GAL1, GAL2, and MEL1). Strain Y187 expresses the *lacZ* reporter gene under the regulation of the GAL1 UAS (James et al., 1996). Both yeast strains were cultured in YPDA agar medium at 30°C. The ORF of the PVF11 gene was cloned into the pGADT7 prey vector and *WRKY* genes were cloned into pGBKT7 bait vectors to induce the expression of proteins that bind to the GAL4 DNA-activating domain containing *LEU2* and *TRP1* reporter genes. The constructed bait and prey vectors were transformed into the yeast strains AH109 and Y187, respectively. Transformants were selected on tryptophan-deficient selective plates (SD/-Trp) and leucine-deficient selective plates (SD/-Leu), respectively (Maier et al., 2008). Mating between the two transformed yeast strains was performed by co-culturing into the same YPDA broth. Successfully mated diploid AH109 were selected on agar plates containing media deficient in leucine, tryptophan, and histidine [(LTH^-) (SD/-His/-Leu/-Trp)] and confirmed growth was made on selective media deficient in leucine, tryptophan, histidine and adenine [($LTHA^-$) (SD/Ade/-His/-Leu/-Trp)] agar plates as a higher stringency assay. The growth of the diploid yeasts within 5 days of incubation was considered indicative of protein-protein interactions (Kolonin et al., 2000).

To test the interaction between PVF11 and rice plant proteins following infection with *B. glumae* BGR1, a genome-wide Y2H screening was performed with PVF11 as the bait and a cDNA library from infected rice as the prey. To prepare the cDNA library, total RNA was extracted with the TRIzol® Reagent (Ambion, Carlsbad, CA, USA) from 3 g of infected rice stems and mRNA was isolated using the Oligotex® mRNA Mini Kit (Qiagen, Hilden, Germany). Reverse transcribed first-strand cDNA was obtained from the mRNA using the Make Your Own Mate & Plate® Library System (Clontech Laboratories Inc., Palo Alto, CA, USA) following the manufacturer's instructions and the amplified double-stranded cDNA was used for the analysis (Gao et al., 2015).

The Y2H assay was performed as follows. Briefly, the complete PVF11 ORF was cloned into a pGBKT7 bait vector and transformed into AH109 yeasts (AH109-pGBKT7-PVF11). The cDNA library was directly introduced into AH109-pGBKT7-PVF11 competent yeast cells via *in vivo* homologous recombination with a pGADT7-Rec prey vector to obtain cDNA fusion to the GAL4 activation domain (Cao and Yan, 2013). Yeast transformants were selected on LTH^- (SD/-His/-Leu/-Trp) agar plates and the surviving colonies, representing candidate positive interactions, were used in a confirmation test to exclude false positives. Target sequences were amplified by long distance PCR (LD-PCR) using Matchmaker 5' AD CTATTCGATGATGAAGATAC, and Matchmaker 3' AD GTGAACTTGCGGGGTTTTTC primers. The LD-PCR was performed using the Advantage® 2 PCR Mix (Clontech Laboratories Inc., Palo Alto, CA, USA) under the following conditions: pre-denaturation at 95°C for 30 s followed by 26 cycles of denaturation at 95°C for 10 s, extension at 68°C for 6 min, and elongation steps increased by 5 s per each successive cycle. An additional extension step was performed at 68°C for 5 min. The obtained distinctive cDNA fragments from candidate positive clones were isolated using Easy Yeast

Plasmid Isolation Kit (Clontech Laboratories Inc., Palo Alto, CA, USA) and re-transformed into *E. coli* DH5α *λpir* competent cells for propagation. cDNA-containing plasmids were co-transformed again with pGBKT7-PVF11 into AH109 yeast competent cells for the confirmation test. The growth of yeast transformants clones on LTH$^-$ (SD/-His/-Leu/-Trp) and LTHA$^-$ (SD/-Ade/-His/-Leu/-Trp) agar plates within 6 days was considered indicative of a positive interaction between PVF11 and the cDNA-encoded protein (Cao and Yan, 2013; Gao et al., 2015). From the positive colonies, the cDNA inserts were amplified and sequenced. Sequences were analyzed using the BLASTn tool of the National Center for Biotechnology Information and the Rice Genome Annotation Project (Michigan State University).

To test the effect of PVF11 on *B. glumae* virulence in different stages and tissues of the rice plants, pathogenicity assays were performed at three stages (seedling, vegetative, and reproductive) of rice *Oryza sativa* L. cv. Saeilmi using *B. glumae* BGR1 wild-type, the PVF11-defective mutant, and the complemented mutant, PVF11C. The seedling assay was performed by immersion of surface-sterilized rice seeds in freshly prepared bacterial suspensions harvested in sterile distilled water (SDW), adjusted to optical density OD_{600nm} = 0.8, and incubated for germination in the dark at 28°C for 2 days. Seeds treated with SDW served as negative controls. Germinated seeds were then transferred to plant culture tubes (SPL Life Sciences, Pocheon, Korea) and incubated in a humid growth chamber at 28°C for 8 days. After incubation, disease severity was examined and the length of the longest shoots and roots was measured.

At the vegetative stage of rice, sterilized toothpicks were used for inoculation by stabbing the rice stems, about 2 mm deep, with a single bacterial colony per stab. Inoculated plants were incubated for 8 days and then collected for assessment of disease severity and bacterial populations. Bacterial populations in inoculated plants were assessed by grinding equal weights of stems (10 cm above the inoculation site) in SDW. The suspensions were then serially diluted and cell population was assessed by a standard spread plate method for counting colony forming units (cfu)/g rice.

At the reproductive stage, the rice panicle assay was conducted as follows. Panicles at the flowering stage, grown under greenhouse conditions, were inoculated by immersion in the bacterial suspensions or SDW control for 1 min. Inoculated plants were then grown for 8 days and disease severity was assessed based on the distribution pattern of the panicle grains, taking into account the degree of grain discoloration on treated panicles (Chun et al., 2009).

Statistical analysis was performed using the Statistical Analysis Systems (SAS Institute, Cary, NC, USA). Analysis of variance was performed using the general linear model in SAS, and means separation determined by the least significant difference (LSD) test with $P < 0.05$.

The preliminary pathogenicity assay of mutant strains defective in each of the 20 candidate PVF genes resulted in the selection of PVF11 for further analysis due to its relative effect on *B. glumae* virulence to rice stem (Supplementary Fig. 1). The PVF11 (bglu_1g30730) is a putative TetR family transcriptional regulator located on chromosome 1 of *B. glumae* BGR1. PVF11 was then

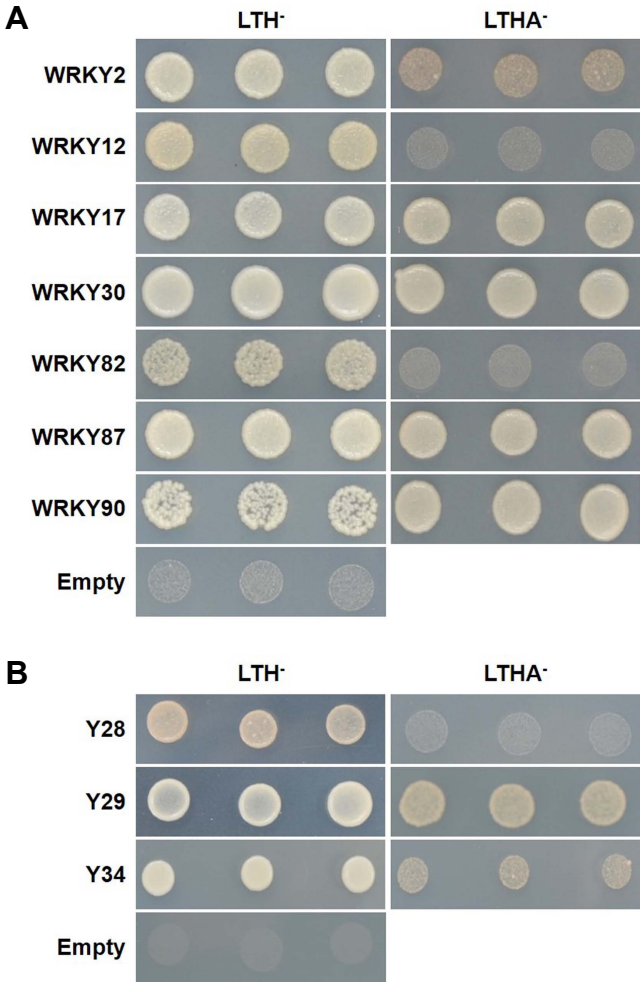

Fig. 1. Yeast two hybrid (Y2H) assays for *Burkholderia glumae* PVF11 interaction with rice defense-related proteins. (A) Positive Y2H interaction between PVF11, as prey, and WRKY transcriptional factors, as baits. The empty bait vector was used as a negative control. (B) Positive Y2H interaction between PVF11, as bait, and rice cDNA libraries (Y28, Y29 and Y34) as prey. In both Y2H assays, positive interactions are indicated by hybridized yeast cell survival on LTH$^-$ (SD/-His/-Leu/-Trp) and LTHA$^-$ (SD/-Ade/-His/-Leu/-Trp) agar plates.

tested for possible interactions with WRKY proteins. The *WRKY* gene superfamily comprises transcription factors implicated in pathways involved in plant defense against pathogen attack in rice and many other plants (Ryu et al., 2006). Possible interactions between pathogen proteins and host WRKY proteins could represent a strategy to escape host defense mechanisms and enhance bacterial virulence. Previous studies reported that mutations in several *WRKY* genes in *Arabidopsis* are linked to reduced resistance to pathogens and that the interaction between specific bacterial effectors and WRKY protein domains results in the inactivation of plant defense and increased bacterial virulence (Birkenbihl et al., 2012; Sarris et al., 2015).

In this study, PVF11 showed positive interactions with 7 of the 12 WRKY transcriptional factors examined in the Y2H assays, i.e., Os01g42850 (encoded by *WRKY2*), Os01g43550 (*WRKY12*), Os05g14370 (*WRKY82*), Os01g74140 (*WRKY17*), Os08g38990 (*WRKY30*), Os07g39480 (*WRKY87*), and Os09g30400 (*WRKY90*). The yeast strains containing both PVF11 and each of the 7 *WRKY* genes exhibited growth on both LTH^- (SD/-His/-Leu/-Trp) and $LTHA^-$ (SD/-Ade/-His/-Leu/-Trp) agar plates, except for those containing *WRKY12* and *WRKY82* genes, which showed slightly reduced growth on $LTHA^-$ plates (Fig. 1A).

After confirmation of the interaction between PVF11 and specific plant defense-related WRKY proteins, the interaction of PVF11 with cDNA library-encoded proteins from BGR1-infected rice was also tested. Y2H assay using cDNA from infected rice revealed a positive interaction of PVF11 with 3 expressed proteins [Y28 (LOC_Os04g57200), Y29 (LOC_Os08g0920), and Y34 (LOC_Os01g12910)] (Fig. 1B). Sequence analysis and gene ontology revealed that the relative genes were associated with the response to biotic and abiotic stress, as well as other related functions in rice (Table 1). In response to infection, generally, plants recognize pathogens and generate defense response proteins. In turn, pathogens utilize virulence effectors physically interacting with host proteins and modulating host defense (Mukhtar et al., 2011). Such continuously evolving dynamic interaction between host and pathogen is the source of variations in pathogen virulence and host resistance.

To test the possible role of PVF11 in *B. glumae* survival and virulence to rice plants, we conducted virulence assays at different stages of rice and found that the PVF11 mutant was associated with reduced virulence at seedling and vegetative stages, compared to wild-type *B. glumae* BGR1. The shoot and root length of wild-type BGR1-infected seedlings was significantly lower than that observed after infection with the PVF11 mutant. Likewise, the bacterial population on stems treated with the PVF11 mutant was significantly lower than in wild-type BGR1-treated plants. The complemented strain, PVF11C, restored the wild-type pattern in both assays (Fig. 2A-D). However, disease severity was not significantly different in rice panicles infected with the PVF11 mutant and wild-type BGR1 (Fig. 2E-F). These results indicated that PVF11 was required for full virulence of *B. glumae* to rice plants at the seedling and vegetative stages but not at the flowering stage.

Identification of virulence factors and characterization of disease-causing mechanisms may help develop strategies to prevent disease spreading and reduce crop losses. Following a comparative transcriptome analysis conducted in *B. glumae* under both *in planta* and *in vitro* conditions, PVF11 was originally selected as one of the genes that are up-regulated during the infection process (Kim et al., 2014). In the present study, the role of PVF11 as a factor interfering with rice biotic and abiotic stress response proteins was confirmed by Y2H analysis, revealing positive interactions of PVF11 with 7 *WRKY* gene-encoded and 3 stress response related proteins from the cDNA library of infected rice. The contribution of PVF11 to *B. glumae* virulence was then confirmed in pathogenicity assays on

Table 1. Putative encoded products and ontology of genes from infected rice that showed positive interaction with PVF11

Clone No.	Locus ID	Gene Product	Gene ontology
Y28	LOC_Os04g57200	Heavy metal transport/detoxification protein, putative, expressed	Metal ion binding and transport Detoxification
Y29	LOC_Os08g09200	Aconitate hydratase protein, putative, expressed	Aconitate hydratase Catalytic activity DNA binding Endogenous stimulus response Biotic and abiotic stress response
Y34	LOC_Os01g12910	Thioesterase family protein, putative, expressed	Hydrolase activity Inhibition to ROS

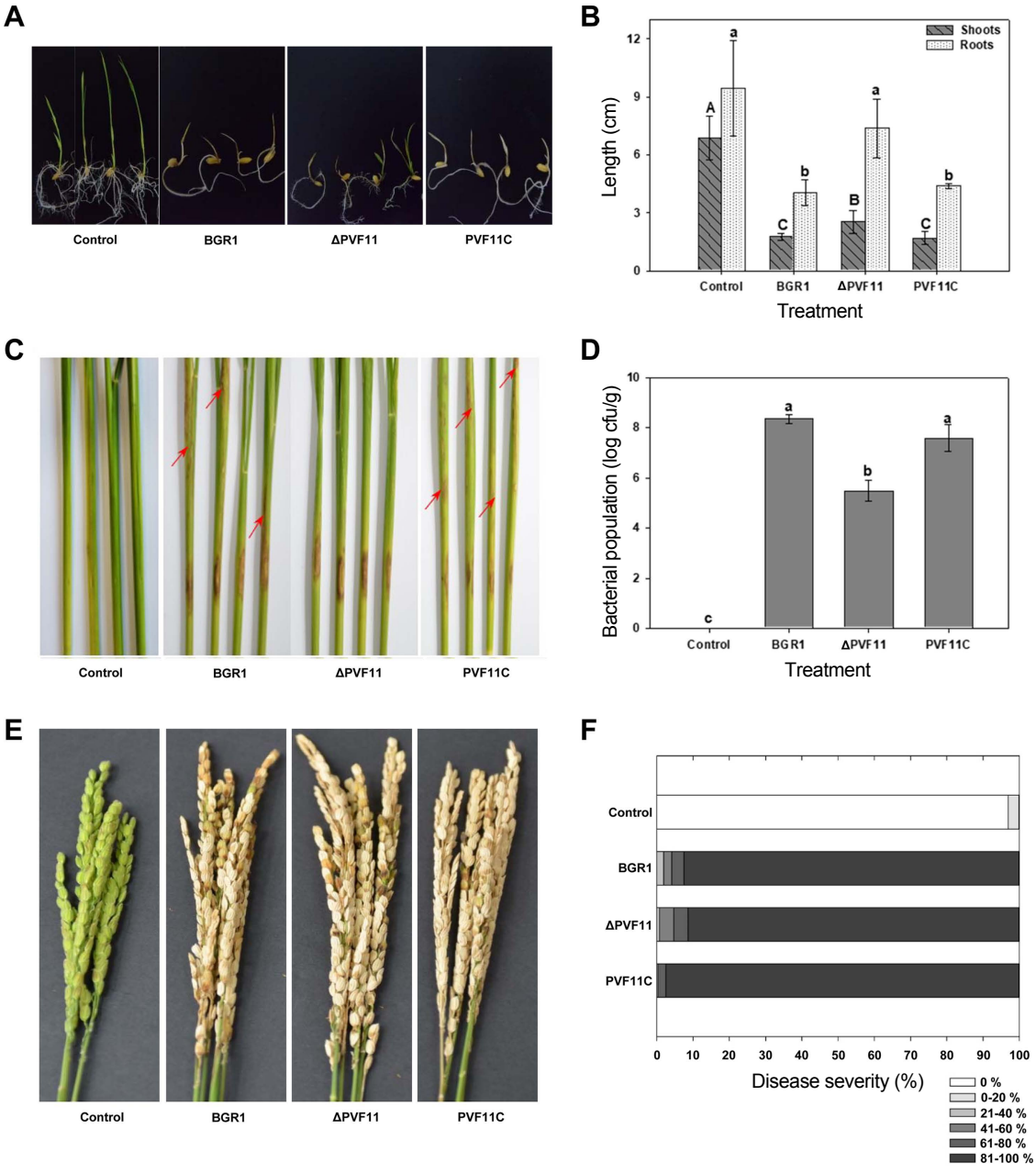

Fig. 2. Pathogenicity assays of *Burkholderia glumae* BGR1 wild-type, PVF11 defective mutant, and PVF11C complemented mutant in rice plants at different growth stages (seedling, vegetative, and reproductive). (A) Photographs from the seedling assay showing a clear deleterious effect of wild-type BGR1 treatment and reduced virulence in the ΔPVF11 mutant-treated seedlings. The complemented mutant, PVF11C, apparently recovered the wild-type virulence. (B) Bar graphs represent the shoot and root lengths as a measure of the bacterial virulence to the seedlings. The different uppercase and lowercase letters on the error bars represent significant differences between shoot and root lengths, respectively, with $P < 0.05$, according to least significant difference test (LSD). (C) Photographs of rice stems at the vegetative stage showing the typical blight symptoms of infected rice stems. Treatment with the ΔPVF11 mutant resulted in reduced virulence as limited symptomatic lesions were observed, compared to plants infected with wild-type BGR1 or the complemented mutant PVF11C. Red arrows show the spread of symptomatic lesions away from the inoculation sites. (D) Bar graphs showing the bacterial populations in treated stems 8 days after inoculation, expressed as log colony forming units (cfu)/g rice stem. The different letters on the error bars indicate significant differences according to LSD test ($P < 0.05$). (E) Photographs from the reproductive stage assay on rice panicles showing the typical blight symptoms on the rice grains after treatment with BGR1, ΔPVF11, and PVF11C. (F) Stacked bar graphs representing disease severity distribution pattern on the harvested grains. There was no difference in disease severity between the wild-type, ΔPVF11, and PVF11C mutants. Sterile distilled water was used as negative control in all assays.

seedling and vegetative stages but not rice panicles. Apparently, the defense mechanisms in rice plant and virulence factors in *B. glumae*, might change depending on the tissue type and the growth stage. Rice plants were able to overcome the interaction between PVF11 and defense-related proteins in the panicles at the flowering stage.

Taken together, our results demonstrated the ability of *B. glumae* to interfere with the host defense machinery via PVF11 as a new virulence factor enhancing host colonization. Moreover, our data showed that the role of PVF11 in virulence to rice was tissue-specific, as increased PVF11-dependent virulence was observed at the seedling and vegetative stage, but not the flowering stage. The latter finding could be related to the activation of distinct defense mechanisms in rice plants and virulence factors from *B. glumae* depending on tissue and growth stage. These results are a step forward toward better understanding and characterization of virulence mechanism in *B. glumae.*

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