The Type III Effector AvrBs2 in *Xanthomonas oryzae* pv. *oryzicola* Suppresses Rice Immunity and Promotes Disease Development

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Xanthomonas oryzae pv. *oryzicola*, the causal agent of bacterial leaf streak, is one of the most important bacterial pathogens in rice. However, little is known about the functions of individual type III effectors in virulence and pathogenicity of *X. oryzae* pv. *oryzicola*. Here, we examined the effect of the mutations of 23 putative nontranscription activator-like effector genes on *X. oryzae* pv. *oryzicola* virulence. The *avrBs2* knock-out mutant was significantly attenuated in virulence to rice. In contrast, the *xopAA* deletion caused enhanced virulence to a certain rice cultivar. It was also demonstrated that six putative effectors, including *XopN*, *XopX*, *XopA*, *XopY*, *XopF1*, and *AvrBs2*, caused the hypersensitive response on nonhost *Nicotiana benthamiana* leaves. Virulence function of *AvrBs2* was further confirmed by transgenic technology. Pathogen-associated molecular pattern-triggered immune responses including the generation of reactive oxygen species and expression of pathogenesis-related genes were strongly suppressed in the *AvrBs2*-expressing transgenic rice lines. Although not inhibiting flg22-induced activation of mitogen-activated protein kinases, heterologous expression of *AvrBs2* greatly promotes disease progression in rice caused by two important bacterial pathogens *X. oryzae* pvs. *oryzae* and *oryzicola*. Collectively, these results indicate that *AvrBs2* is an essential virulence factor that contributes to *X. oryzae* pv. *oryzicola* virulence through inhibiting defense responses and promoting bacterial multiplication in monocot rice.

Plants possess multiple layers of preformed and induced defenses to protect themselves from pathogen attack (Jones and Dangl 2006). During plant-pathogen coevolution, pattern recognition receptors (PRR) in plants recognize pathogen-associated molecular patterns (PAMPs) such as bacterial flagellin and fungal chitin and, thus, trigger the first layer of defense responses, called PAMP-triggered immunity (PTI). PTI includes a series of defense signal transduction cascades that lead to callose deposition, bursts of reactive oxygen species (ROS), induced expression of pathogenesis-related (*PR*) genes, phytoalexin accumulation, and the deposition of phenolic

compounds (Chisholm et al. 2006; Jones and Dangl 2006). Many gram-negative phytopathogenic bacteria, such as *Pseudomonas* and *Xanthomonas* spp., translocate a large number of type III effectors (T3E) into the host cells through the type III secretion system (Kay and Bonas 2009). The secreted T3E often target essential components in the PTI signaling pathway, such as PRR, mitogen-activated protein kinases (MAPK), and transcription factors to interfere with or suppress the plant immune system (Boller and He 2009; Dou and Zhou 2012; Xin and He 2013). Accordingly, plants have evolved to specifically recognize certain T3E by the intracellular cognate resistance (*R*) proteins to initiate the second layer of defenses, referred to as effector-triggered immunity (ETI). As compared with PTI, ETI is more robust and effective and is often characteristic of the hypersensitive response (HR) at the invasion site that inhibits pathogen multiplication (Chisholm et al. 2006; Jones and Dangl 2006).

As a major group of virulence factors, the T3E and their secretion apparatus encoded by the hypersensitive reaction and pathogenicity (*hrp*) gene cluster are essential for pathogenesis and virulence of phytopathogenic bacteria (Alfano and Collmer 2004; Kay and Bonas 2009). In *Xanthomonas* spp., *HrpX* is a key *hrp* regulatory factor and regulates expression of some T3E genes through binding to the PIP (plant-inducible promoter) boxes (Furutani et al. 2006; Li et al. 2011). The PIP box is a conserved *cis* element consisting of the consensus sequence TTCGB-N15-TTCGB ('B' represents any base except adenine) that is located at 30 to 32 bases upstream of the start codon of the effector gene (Furutani et al. 2006; Koebnik et al. 2006; Tsuge et al. 2006). The T3E usually carry a secretion signal at their N-terminus, which often contains 50 specific amino-acid residues (Alfano and Collmer 2004; Cunnac et al. 2004; Tampakaki et al. 2004).

The T3E in xanthomonads, also called *Xanthomonas* outer proteins (*Xops*), are categorized into up to 39 protein families based on sequence similarity, reflecting genetic diversity of the virulence factor inventory in the pathogens (White et al. 2009). A group of T3E unique to *Xanthomonas* spp. are called transcription activator-like (TAL) effectors (Scholze and Boch 2011) and others are grouped into non-TAL effectors (Furutani et al. 2009; White et al. 2009). Various biological functions of T3E in phytopathogenic bacteria, especially in *Pseudomonas* spp., have been recently revealed in succession (Xin and He 2013). Some effectors, such as *AvrPtoB*, function as E3 ubiquitin ligases and promote the degradation of immunity-related proteins in a proteasome-dependent manner (Rosebrock et al. 2007). Some effectors, such as the *YopJ/AvrRxv* family, target the proteasomal subunit to inhibit the proteasome activity (Üstün et al. 2013). Some effectors, such as *HopAO1*, alter the phosphorylation state

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of host proteins (Li et al. 2013; Underwood et al. 2007). Other effectors, especially for TAL effectors including the AvrBs3/PthA family, act as transcription factors that regulate expression of susceptibility genes in hosts and promote bacterial spreading and multiplication (Boch and Bonas 2010; Pereira et al. 2014).

In recent years, intensive research has been performed to study functions of the Xop proteins. Besides TAL effectors, some non-TAL effector genes, including *xopR*, *xopN* and *xopZ*, have been demonstrated to be required for full virulence in *X. oryzae* pv. *oryzae* (Akimoto-Tomiya et al. 2012; Sinha et al. 2013; Song and Yang 2010). The *X. campestris* pv. *vesicatoria* effector XopX contributes to *X. campestris* pv. *vesicatoria* virulence in host pepper and tomato plants (Metz et al. 2005). Several host targets of the Xops have been identified recently. XopAA and XopY in *X. oryzae* pv. *oryzae* interact with OsSERK1/2 and OsRLCK185 to inhibit rice immunity, respectively (Yamaguchi et al. 2013a and b). The XopN homologs target a zinc finger domain-containing protein, OsVOZ2, and a putative thiamine synthase, OsXNP, in rice (Cheong et al. 2013) and the atypical receptor-like kinase TARLK1 in tomato to promote pathogenicity (Kim et al. 2009). In *X. campestris* pv. *vesicatoria*, XopD functions as a SUMO protease and interacts with the transcription factor SIERF4, to inhibit ethylene production and to promote pathogen growth in tomato (Kim et al. 2008, 2013). It has recently been shown that XopQ in *X. campestris* pv. *vesicatoria* suppresses ETI by targeting the tomato 14-3-3 isoform SITFT4, an important component of ETI (Teper et al. 2014). The effector AvrAC secreted by *X. campestris* pv. *campestris* functions as a uridylyl transferase that transfers uridine 5'-monophosphate to and masks conserved phosphorylation sites in the activation loop of BIK1 and RIPK, thus preventing activation of these kinases and suppressing defense signaling in host (Feng et al. 2012). Most recently, XopD in *X. campestris* pv. *campestris* has been demonstrated to target DELLA proteins in *Arabidopsis* to trigger disease tolerance and increase bacterial survival (Tan et al. 2014).

Bacterial leaf streak (BLS), caused by *X. oryzae* pv. *oryzicola*, is currently one of the most serious bacterial diseases in rice and causes significant yield loss in rice-growing regions of South Asia and South China (Niño-Liu et al. 2006). Due to similar biochemical characteristics and disease symptoms on rice leaves, the disease was previously considered to be bacterial blight. Subsequent studies revealed significant differences in infection style and pathogenicity between *X. oryzae* pvs. *oryzae* and *oryzicola* (Bogdanove et al. 2011; Vauterin et al. 1995). *X. oryzae* pv. *oryzicola* enters and colonizes the intercellular spaces between mesophyll cells in rice leaves through stomata or wounds. In contrast, *X. oryzae* pv. *oryzae* infects rice leaves through vascular tissues and causes a systemic disease (Niño-Liu et al. 2006). Therefore, molecular mechanisms underlying virulence and pathogenicity might be significantly different between *X. oryzae* pvs. *oryzae* and *oryzicola*. The inventories of effector proteins in many *Xanthomonas* species have been predicted from publicly available genome sequences of these species (The Xanthomonas Resource database) (Bogdanove et al. 2011). As mentioned above, functional studies on Xops focus on only a few *Xanthomonas* species, including *X. oryzae* pv. *oryzae* and *X. campestris* pvs. *campestris*, and *vesicatoria*. As compared with ever-increasing reports on *X. oryzae* pv. *oryzae* effectors, little is known about the functions of *X. oryzae* pv. *oryzicola* effector proteins, particularly for non-TAL effectors.

AvrBs2, the first reported non-TAL effector in *X. campestris* pv. *vesicatoria* (Kearney and Staskawicz 1990), is highly conserved in xanthomonads (Swords et al. 1996). AvrBs2 was characterized as an avirulence protein that is recognized by the R protein Bs2 in pepper (Gassmann et al. 2000; Tai et al. 1999). Computational and biochemical evidence showed that AvrBs2 contains a glycerophosphodiesterase catalytic domain that is

essential for its virulence function but not for activation of Bs2-mediated disease resistance (Zhao et al. 2011). Functional studies on AvrBs2 have been focused on the dicot plants. In contrast, no experiment has been performed so far to determine whether *avrBs2* in *X. oryzae* pv. *oryzicola* is required for bacterial virulence to the monocot rice.

In this study, we performed mutational analyses of 23 putative Xop genes in the *X. oryzae* pv. *oryzicola* RS105, revealing that the effector gene *avrBs2* is required for full virulence of *X. oryzae* pv. *oryzicola*. It was also demonstrated that multiple effectors including XopN, XopA, XopX, XopF1, XopY, and AvrBs2 triggered HR in nonhost *N. benthamiana* to different degrees. Ectopic expression of AvrBs2 in transgenic rice plants inhibited defense responses including flg22- and chitin-triggered ROS burst and *PR* gene expression and, importantly, promoted disease progression after infection by rice bacterial pathogens.

RESULTS

Construction of gene-deletion mutants for 23 putative non-TAL effector genes in *X. oryzae* pv. *oryzicola*.

The effector genes in *X. oryzae* pv. *oryzicola* have been predicted based on the available genome sequence of *X. oryzae* pv. *oryzicola* BLS256 (Bogdanove et al. 2011). Among them, at least 26 genes were predicted to encode non-TAL effectors. The degree of conservation of these effectors was predicted and compared among *Xanthomonas* species (Supplementary Fig. S1), and the location of these genes in the genome and the sequences of the PIP boxes were also identified (Supplementary Table S1). The majority of non-TAL effectors are highly conserved, although some effector genes have presence/absence polymorphisms in *Xanthomonas* species (Bogdanove et al. 2011). A total of 11 non-TAL effector families are widely distributed in genome sequence-available *Xanthomonas* species and, thus, are considered as core effector proteins in xanthomonads. A few effector genes including *xopU*, *xopW*, *xopY*, and *xopAB* are present exclusively in *Xanthomonas oryzae* pathovars. The *xopT* and *xopAF* genes only exist in *X. oryzae* pvs. *oryzae* and *oryzicola*, respectively. The *xopO* and *xopAJ* genes exist in both *X. oryzae* pvs. *oryzicola* and *vesicatoria*. Among the 26 non-TAL effectors, the only XopAF is unique to *X. oryzae* pv. *oryzicola*.

To initially understand the roles of putative non-TAL effector genes in *X. oryzae* pv. *oryzicola* virulence, marker-free deletion mutants of the 23 effector genes were constructed via homologous recombination as described below. The *xopA* and *hpaA* were excluded in mutational analyses, since they most likely encode a harpin and a type III system control protein, respectively, which might not be T3E, and *xopAJ*, also called *avrXo1*, was not selected because of failure to make the *avrXo1* knock-out mutant in a previous study (Zhao et al. 2004). The mutant strains were used for subsequent functional studies after being confirmed through Southern blot analyses (Supplementary Figs. S2 and S3).

The mutations of *avrBs2* and *xopAA* cause altered virulence of *X. oryzae* pv. *oryzicola* to rice.

To determine whether the putative effector genes are required for full virulence of *X. oryzae* pv. *oryzicola* to rice, all constructed marker-free gene-deletion mutants were pressure-inoculated into rice leaves for virulence assays. Virulence of each mutant strain was evaluated by the measured length of disease lesions at 2 weeks after inoculation compared with that of the wild-type strain. The results demonstrated that all gene-deletion mutants except Δ *avrBs2* and Δ *xopAA* exhibited no or little alteration in *X. oryzae* pv. *oryzicola* virulence under the experimental conditions used in this study (Fig. 1A). Disease lesions in the Δ *avrBs2*-inoculated leaves were much shorter than those caused

by the wild-type strain (Fig. 1A; Supplementary Fig. S4). The complementation strain with the plasmid-borne full-length *avrBs2* gene largely restored virulence of the Δ *avrBs2* mutant toward the wild-type level. Enumeration of bacteria extracted from inoculated rice leaves clearly showed that the in planta population size of Δ *avrBs2* was much smaller than that of the wild-type and complementation strains during infection (Fig. 1B). Taken together, the data indicate that *AvrBs2* is one of the major virulence factors and plays an essential role in virulence and multiplication of *X. oryzae* pv. *oryzicola* in rice. Interestingly, the Δ *xopAA* mutant caused significantly longer disease lesions on the leaves of rice cv. Jingang 30 than the wild-type strain (Fig. 1A). The population size of Δ *xopAA* was also larger than that of the wild-type strain in inoculated rice leaves 8 days after inoculation (Fig. 1C). Introduction of the plasmid-borne *xopAA* gene reduced virulence of the Δ *xopAA* mutant to the wild-type level (Fig. 1A and C). Virulence of the *xopAA* mutant to rice was further tested using the other two rice cultivars, Nipponbare and Jinhe 2. The

results showed that the *xopAA* mutant and wild-type strains caused almost equal length of disease lesions on these cultivars (Supplementary Fig. S5). The data suggest that *XopAA* in *X. oryzae* pv. *oryzicola* triggers defense responses in a certain rice genetic background.

The HR triggered by putative *X. oryzae* pv. *oryzicola* effectors on nonhost *N. benthamiana* leaves.

X. oryzae pv. *oryzicola* causes nonhost hypersensitive cell death in *N. benthamiana*. In order to investigate which effector candidates have cell death-eliciting activities in *N. benthamiana*, 22 putative effectors, not including *XopAB*, *XopZ1*, *XopAA*, and *XopO*, were transiently expressed in *N. benthamiana* by *Agrobacterium tumefaciens*-mediated expression system. The cell-death symptoms were monitored within 3 days after treatment with dexamethasone (DEX), which induced expression of these effector genes. The infiltrated leaf area expressing *XopN* became necrotic at 1 day after DEX treatment. Expression of

Fig. 1. The effects of mutation of each putative effector gene on virulence of *Xanthomonas oryzae* pv. *oryzicola* to rice. **A**, Mutation of *avrBs2* caused a significant reduction in virulence, while the Δ *xopAA* mutant exhibited an enhanced virulence on rice cv. Jingang 30 in repeatable tests. Virulence of the Δ *avrBs2* and Δ *xopAA* mutants was successfully complemented with plasmid-borne full-length *avrBs2* and *xopAA* genes, respectively. Virulence of each mutant was evaluated by the length of disease lesions after bacteria were inoculated into rice leaves by pressure infiltration compared with that caused by the wild-type (WT) strain. The length of disease lesions was measured at 14 days after inoculation for 10 to 15 leaves per strain. The gene-deletion mutants were constructed for 23 of 26 putative effector genes that were all present in the *X. oryzae* pv. *oryzicola* RS105 strain. **B**, In planta bacterial population of *X. oryzae* pv. *oryzicola* RS105, Δ *avrBs2*, and Δ *avrBs2*(*avrBs2*) at the indicated days postinoculation (dpi). **C**, In planta bacterial population of *X. oryzae* pv. *oryzicola* RS105, Δ *xopAA*, and Δ *xopAA*(*xopAA*) at the indicated time points after inoculation. Bars are means $\pm$ standard error. Different letters a to d indicate statistically significant difference ($P < 0.05$). Δ *avrBs2*(*avrBs2*) and Δ *xopAA*(*xopAA*) are the complementation strains of Δ *avrBs2* and Δ *xopAA*, respectively.

XopX and XopA caused similar HR symptoms at approximately 2 days, while XopF1, XopY, and AvrBs2 induced cell-death phenotypes at approximately 3 days. XopN and XopX caused HR on all of 20 inoculated leaves, while XopA, XopY, XopF1, and AvrBs2 induced HR to relatively lower degrees (Fig. 2A). Other tested putative effectors did not trigger cell-death symptoms in *N. benthamiana*, although expression of those proteins was all confirmed by Western blotting (Fig. 2B).

Flg22-induced immunity is suppressed by transient expression of AvrBs2 in *Arabidopsis* protoplasts.

The expression of *NONHOST1* (*NHO1*), an essential gene for nonhost resistance, is induced by flg22 in *Arabidopsis*. It has been well demonstrated that flg22-induced *NHO1* expression was inhibited by different T3E in *Arabidopsis* protoplasts (Li et al. 2005; Lu et al. 2001). Transient expression assays in *Arabidopsis* protoplasts were used herein to investigate if AvrBs2 can suppress flg22-induced *NHO1* expression. Protoplasts isolated from the *NHO1*-luciferase (*NHO1-LUC*) transgenic *Arabidopsis* plants were transfected with an empty vector and the *hopA11* construct as negative and positive controls,

Fig. 2. Cell death symptoms on *Nicotiana benthamiana* leaves caused by transient expression of *Xanthomonas oryzae* pv. *oryzicola* putative effectors. **A**, Six proteins of the 22 tested putative effectors, i.e., XopN, XopX, XopA, XopY, XopF1, and AvrBs2, caused the hypersensitive response in *N. benthamiana* to different degrees. XopQ, which did not induce HR, was shown as a negative control. *Agrobacterium* cultures transformed with different effector constructs were infiltrated into *N. benthamiana* leaves, followed by dexamethasone (DEX) spraying. Representative photos were taken at 3 days after DEX treatment. Numbers in parentheses, e.g., 18/20, indicate that 18 of 20 inoculated leaves exhibited cell-death symptoms. CK, empty vector. **B**, Transient expression of 22 tested putative effectors in *N. benthamiana* was confirmed via Western blot analyses. The samples for protein extraction were collected before cell-death symptoms were visible. The effectors with the FLAG tag were detected by immunoblotting with an anti-FLAG antibody.

respectively. As shown in Figure 3A, LUC expression driven by the *NHO1* promoter was greatly induced by flg22 in protoplasts transfected with empty vector. Transfection of the *avrBs2* construct nearly abolished flg22-induced LUC expression, which was similar to transfection of *hopA11*. These data suggest that transient expression of AvrBs2 in protoplasts strongly suppresses flg22-induced expression of defense genes.

In order to determine subcellular localization of AvrBs2 in host cells, the green fluorescent protein (*GFP*) gene was fused in frame with the *avrBs2* coding sequence at its C terminus in the pUC19 plasmid with the 35S promoter. The recombinant AvrBs2-GFP protein was transiently expressed in rice protoplasts. Green fluorescence of AvrBs2-GFP was detected across the entire cell, which is similar to subcellular localization of AvrBs2-GFP in *N. benthamiana* (Fig. 3B; Supplementary Fig. S6). Transient expression of AvrBs2-GFP in *N. benthamiana* caused HR symptoms, indicating that the AvrBs2-GFP fusion protein is functional (data not shown).

Ectopic expression of AvrBs2 suppresses PTI signaling in rice cell cultures.

To investigate if AvrBs2 suppresses plant immunity in rice, the transgenic rice cell cultures with heterologous expression of AvrBs2-FLAG driven by the DEX-inducible promoter was generated in the genetic background of rice cv. Nipponbare. Induced expression of *avrBs2-FLAG* in the transgenic rice cells was confirmed by quantitative real time reverse transcription-polymerase chain reaction (RT-PCR) (data not shown) and

Fig. 3. Transient expression of AvrBs2 inhibits flg22-induced *NHO1-LUC* expression in *Arabidopsis* protoplasts. **A**, The relative luciferase (LUC) activity in transfected *Arabidopsis* protoplasts. Protoplasts were transfected either with the *avrBs2* gene construct or empty vector (EV). The relative LUC activity was measured 12 h after flg22 treatment. EV- indicates EV-transfected protoplasts treated with distilled H₂O, which was used as a control for basal *NHO1-LUC* expression. EV+ represents EV-transfected protoplasts treated with 1 μ M flg22. Each data point (mean $\pm$ standard error) consists of ten replicates. The experiments were repeated at least three times with similar results. Asterisks (**) indicates *P* value < 0.01. **B**, Subcellular localization of AvrBs2-GFP transiently expressed in rice protoplasts. The empty vector pUC19-35S-GFP was used as a control.

immunoblotting (Fig. 4A). As virulence factors, the effectors secreted by plant pathogens often suppress defense responses including PAMP-triggered ROS burst, *PR* gene expression, and MAPK activation (Boller and He 2009; Dou and Zhou 2012). Previous studies revealed that rice senses the PAMPs flg22 and chitin through OsFLS2 (Takai et al. 2008) and through synergetic action of CEBiP and CERK1 (Shimizu et al. 2010), respectively. Here, we measured ROS accumulation in the *avrBs2* transgenic rice cell cultures after DEX treatment followed by flg22 and chitin stimulation, using a highly sensitive ROS detection method. ROS bursts were clearly detected in the transgenic cell cultures with mock treatment in response to flg22 and chitin (Fig. 4B and C). In contrast, ROS accumulation induced by flg22 was reduced up to approximately 60% and chitin-induced ROS was inhibited even more dramatically in the *avrBs2* transgenic cell cultures after DEX treatment (Fig. 4B and C). Furthermore, the defense marker genes *OsPBZ1* and *OsPAL1* were demonstrated to be up-regulated by flg22 and chitin in the transgenic rice cell cultures with mock treatment. Remarkably, induced expression of the two defense genes was almost completely blocked in the transgenic rice cells after DEX-induced expression of AvrBs2 (Fig. 4D and

E). These data indicate that AvrBs2 contributes to *X. oryzae* pv. *oryzicola* virulence by suppressing PAMP-triggered defense responses in rice.

Ectopic expression of AvrBs2 suppresses PTI signaling and promotes disease progression in rice plants.

To further confirm virulence functions of *X. oryzae* pv. *oryzicola* AvrBs2 in suppressing rice immunity, stably transformed transgenic rice plants with DEX-inducible expression of AvrBs2-FLAG were created. Among eight independent transgenic lines, the two T2 homozygous lines A4 and A5 were selected for subsequent functional studies. The transgenic A5 line with a low leaked expression had a lower expression level of AvrBs2 than the A4 line after DEX treatment (Fig. 5A). Consistent with the results in transgenic cell cultures, we showed that flg22 and chitin induced expression of *OsPBZ1* and *OsPAL1* in the mock-treated transgenic plant leaves and the induction was dramatically suppressed by DEX-induced expression of AvrBs2 (Fig. 5B and C). In contrast, MAPK activation induced by flg22 or chitin was not significantly altered by AvrBs2 expressed in the transgenic plants after DEX treatment (Supplementary Fig. S7).

Fig. 4. Heterologous expression of AvrBs2 inhibits pathogen-associated molecular pattern-induced reactive oxygen species (ROS) burst and defense gene expression in transgenic rice culture. **A**, Expression of AvrBs2 in transgenic rice cell culture induced by dexamethasone (DEX) treatment. The AvrBs2-FLAG fusion was detected by Western blotting with an anti-FLAG antibody. NPB = Nipponbare; – = without DEX treatment; + = with DEX treatment; WB = Western blot. Ponceau S staining shows the equal loading of the total proteins. **B** and **C**, ROS burst induced by flg22 and chitin, respectively, was significantly suppressed in the *avrBs2* transgenic cell culture after DEX treatment. ROS generation was detected by a luminol-chemiluminescence assay. **D** and **E**, Upregulation of the defense marker genes *OsPBZ1* and *OsPAL1*, respectively, induced by flg22 and chitin was almost completely blocked in the *avrBs2* transgenic cell culture after DEX treatment. The transgenic cell culture was treated with 10 μ M DEX or 0.03% ethanol as mock control for 24 h, followed by the treatment of 1 μ M flg22 or chitin. Asterisks (**) indicate *P* value < 0.01; means $\pm$ standard error are shown.

It is important to investigate whether AvrBs2 expression in the transgenic plants promotes disease development during the infection of bacterial pathogens. Bacterial disease susceptibility in the *avrBs2* transgenic plants was first evaluated after *X. oryzae* pv. *oryzicola* inoculation. Disease symptoms observed on the inoculated leaves showed that disease lesions in transgenic rice lines A4 and A5 were significantly longer after DEX spraying, as compared with those under mock treatment. The lesion length on the A4 transgenic line was longer than that on the A5 line, which is consistent with the higher AvrBs2 expression level in the A4 line after DEX induction (Fig. 5A and D; Supplementary Fig. S8).

Bacterial growth curve assays demonstrated that the population size of *X. oryzae* pv. *oryzicola* in the transgenic lines upon DEX treatment was also significantly higher than that in the wild-type plant at 12 days after pathogen inoculation (Fig. 5E). Meanwhile, these transgenic plants, when treated with DEX, also exhibited more severe disease symptoms with longer disease lesions than the wild-type plants after *X. oryzae* pv. *oryzicola* inoculation (Fig. 5F). Collectively, these results indicate that AvrBs2 expression in the transgenic rice plants disrupts plant basal defenses during the compatible interaction and, thus, promotes bacterial multiplication and disease symptom development.

Fig. 5. Heterologous expression of AvrBs2 suppresses rice immunity and promotes disease progression caused by bacterial pathogens in transgenic rice plants. **A**, Induced expression of AvrBs2 in the T2 transgenic homozygous lines A4 and A5 after dexamethasone (DEX) treatment. The AvrBs2-FLAG fusion was detected by Western blotting with an anti-FLAG antibody. WT = the wild-type rice; – = without DEX treatment; + = with DEX treatment; WB = Western blot. Ponceau S staining shows the equal loading of the total proteins. **B** and **C**, Upregulation of the defense marker genes *OsPBZ1* and *OsPAL1*, respectively, induced by flg22 and chitin was almost completely inhibited in the *avrBs2* transgenic A4 and A5 lines upon DEX treatment. The rice leaves were sprayed with 30 μ M DEX or 0.1% ethanol as mock control, followed by incubation with flg22 or chitin for 6 h. **D**, The length of disease lesions caused by the *Xanthomonas oryzae* pv. *oryzicola* RS105 in the wild-type Nipponbare and *avrBs2* transgenic lines A4 and A5. **E**, In planta bacterial population of RS105 in the wild type and *avrBs2* transgenic lines A4 and A5 at the indicated time points after inoculation. **F**, The length of disease lesions caused by *X. oryzae* pv. *oryzae* PXO99A in the wild type and *avrBs2* transgenic lines A4 and A5. The wild-type and *avrBs2* transgenic lines were inoculated with *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae* at 24 h after spraying with 30 μ M DEX and 0.1% ethanol as mock control. Asterisks (*) and (**) indicate *P* values < 0.05 and 0.01, respectively; means $\pm$ standard error are shown.

DISCUSSION

Rice, one of the staple food crops, is subject to infection of various pathogenic microbes. *X. oryzae* pv. *oryzicola* causes BLS, which is currently one of the most serious bacterial diseases in rice and is a great threat to commercial production (Xue et al. 2014). Breeding for disease resistance in rice is considered to be the most effective, environmentally friendly approach to controlling the disease. However, no endogenous BLS resistance gene has been identified in rice so far, although a bunch of resistance genes are available for bacterial blight (Niño-Liu et al. 2006). The lack of BLS-resistant germplasm in rice might be partially explained by the finding that *R* gene-mediated resistance is suppressed by the T3E secreted by *X. oryzae* pv. *oryzicola* (Makino et al. 2006). Therefore, it is important to study the effect of individual effectors on *X. oryzae* pv. *oryzicola* virulence for better understanding of molecular mechanisms underlying virulence and pathogenicity of *X. oryzae* pv. *oryzicola*. In this study, we investigated the contribution of each non-TAL effector gene to *X. oryzae* pv. *oryzicola* virulence in a genome-wide analysis. Among 23 tested gene-deletion mutants of the *X. oryzae* pv. *oryzicola* RS105 strain, the *avrBs2* mutation is the only one that leads to reduced virulence of *X. oryzae* pv. *oryzicola* to rice, while the deletion of *xopAA* enhanced *X. oryzae* pv. *oryzicola* virulence exclusively to rice cv. Jingang 30. It is notable that the mutational study did not include the previously identified effector genes *hrpE3* and *Xrp5*, which have been demonstrated to be required for full virulence of *X. oryzae* pv. *oryzicola* (Cui et al. 2013; Xue et al. 2014). Meanwhile, we revealed that six putative effectors caused the HR in nonhost *N. benthamiana* plants through *Agrobacterium*-mediated transient gene expression.

Most of the mutations of individual non-TAL effector genes investigated here did not alter *X. oryzae* pv. *oryzicola* virulence, which is consistent with previous reports on plant bacterial pathogens. No non-TAL effectors but one TAL effector were identified to be required for *X. oryzae* pv. *oryzicola* virulence through a large-scale screening of a Tn5-tagged *X. oryzae* pv. *oryzicola* mutant library. In contrast, the study showed that many *hrp* genes are essential for its virulence (Guo et al. 2012). Virulence assays of the deletion mutants of 18 individual Xop genes in *X. oryzae* pv. *oryzae* revealed that XopZ is the only effector that is required for full virulence of *X. oryzae* pv. *oryzae* in the susceptible host plant (Song and Yang 2010). Similarly, only the *xopN* mutation, out of seven effector genes tested in *X. campestris* pv. *vesicatoria*, has a detectable phenotypic effect on the pathogenicity (Roden et al. 2004). In addition, the mutants with disruption of eight *X. campestris* pv. *campestris* Xop genes individually or collectively did not exhibit any alteration in virulence compared with the wild-type strain on susceptible host plants (Castañeda et al. 2005). Experimental studies on other phytopathogenic bacteria also revealed that individual effector genes are often dispensable for pathogenesis of *Pseudomonas syringae* and *Ralstonia solanacearum* (Cunnac et al. 2004; Kvitko et al. 2009). Function redundancy of multiple effectors in one pathogenic organism is speculated to be a major factor that prevents detecting obvious phenotypic effect of individual effector mutations on host responses to pathogen infection. The hypothesis is supported by the findings that the same host targets have been identified for different sets of effectors in plant bacterial pathogens (Xin and He 2013). For instance, it was demonstrated that both of the effectors HopZ1a and HopX1 in *P. syringae* with different biochemical functions target to and promote degradation of JAZ proteins and contribute to bacterial virulence (Gimenez-Ibanez et al. 2014; Jiang et al. 2013). On the other hand, we cannot rule out

another explanation. The effect on *X. oryzae* pv. *oryzicola* virulence of individual gene deletions might not be detected under experimental conditions used in this study. Some effectors have been shown to suppress preinvasive immunity, such as stomatal closure (Lozano-Durán et al. 2014). However, the pressure infiltration method used here for evaluating *X. oryzae* pv. *oryzicola* virulence most likely bypasses the first layer of rice immunity, such as stomatal defenses, which might be triggered before or during the natural entry of the pathogen into host extracellular spaces (Zeng et al. 2011).

Many effector proteins secreted by plant pathogens are known to have multiple activities, e.g., functioning as a virulence factor in the susceptible host plants, acting as an avirulence protein in the presence of its cognate resistance protein, or triggering HR in the nonhost plants (Göhre and Robatzek 2008). Here, we investigated the HR-eliciting activity of most of the *X. oryzae* pv. *oryzicola* putative effectors in the nonhost *N. benthamiana* plants using a heterogeneous expression system and revealed that at least six putative effectors, i.e., XopN, XopX, XopA, XopY, XopF1, and AvrBs2, caused the nonhost HR (Fig. 2). Among them, XopN, XopX, XopY, and AvrBs2 have been demonstrated to suppress plant immunity in other *Xanthomonas* species (Kim et al. 2009; Metz et al. 2005; Tai et al. 1999; Yamaguchi et al. 2013b). However, the functions of these effectors in *X. oryzae* pv. *oryzicola* virulence need to be further elucidated.

Interestingly, the *xopAA* deletion mutant was found to have an enhanced virulence to rice cv. Jingang 30, suggesting that XopAA secreted by *X. oryzae* pv. *oryzicola* induces immune responses in rice during infection (Fig. 1). A similar phenomenon has been reported for the *xopQ* mutant of *X. oryzae* pv. *oryzicola*, which exhibited an elevated virulence to rice cv. IR28 (Pei et al. 2010). We also demonstrated that the *xopAA* mutant was not more virulent than the wild-type strain to other rice cultivars. In addition, XopAA is relatively less conserved and is missing in *X. campestris* pv. *campestris* and *X. campestris* pv. *citri* as compared with core effectors in xanthomonads. Therefore, XopAA is less likely recognized as a conserved signature given that PAMPs and PTI signaling are usually highly conserved within species (Thomma et al. 2011). Presumably, XopAA is recognized as an avirulence protein and the R-Avr protein interaction is partially suppressed by virulence factors since no incompatible rice-*X. oryzae* pv. *oryzicola* interaction has been observed so far. Otherwise, XopAA might target negative regulators of rice defense signaling to initiate disease tolerance, which has been recently reported for XopD in *X. campestris* pv. *campestris* (Tan et al. 2014). Identification of one or more host targets of the effector XopAA will help to explain the phenomenon.

On the other hand, bacterial effector proteins often suppress PTI and, thereby, enhance pathogenesis in their host plants during the compatible interaction (Göhre and Robatzek 2008). Virulence assays revealed that *avrBs2*, among the 23 investigated effector genes, is the only one for which mutation caused a significant attenuation effect on *X. oryzae* pv. *oryzicola* virulence. The finding is interesting, since a previous mutagenesis study demonstrated that the mutation of *avrBs2* did not significantly alter *X. oryzae* pv. *oryzae* virulence (Song and Yang 2010) and the *avrBs2* genes in *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* share 97% coding sequence identity. Similarly, it was previously reported that the *rpfG* mutants of *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzicola* have different virulence-associated phenotypes (Zhang et al. 2013). Distinct infection processes might cause different molecular mechanisms underlying virulence and pathogenicity among *Xanthomonas* species. In addition, genetic redundancy in the effector inventory of *X. oryzae* pv. *oryzae* and diverse genetic background of rice cultivars could also explain this phenomenon. It was reported that the same *X. oryzae*

pv. *oryzae* strain might cause different phenotypes on different rice cultivars (Sinha et al. 2013). Subsequently, virulence functions of AvrBs2 were further confirmed using transient expression and transgenic technology. Ectopic expression of pathogen effectors in host plants has been widely used to elucidate virulence functions of bacterial and fungal effectors (Hauck et al. 2003; Park et al. 2012). *NHO1* in *Arabidopsis* is essential for preventing the in planta growth of nonhost *Pseudomonas* bacteria (Lu et al. 2001). It is well known that flg22 induces the expression of *NHO1* (Li et al. 2005). We demonstrated that AvrBs2 transiently expressed in *Arabidopsis* protoplasts suppressed flg22-induced *NHO1* expression (Fig. 3A). Furthermore, induced expression of AvrBs2 in transgenic cell cultures was shown to dramatically suppress flg22-induced and chitin-induced immune responses, such as ROS burst and *PR* gene expression (Fig. 4). Third, the AvrBs2-expressing transgenic rice plants displayed a nearly complete suppression of defense gene expression triggered by the PAMPs flg22 and chitin. Most importantly, heterologous expression of AvrBs2 in the transgenic plants facilitated bacterial colonization and multiplication in planta and accelerated disease progression (Fig. 5). The phenomenon that the AvrBs2-expressing transgenic rice is more susceptible to *X. oryzae* pv. *oryzicola* than is the wild-type plant is likely due to the high expression level of AvrBs2 after DEX induction or expression timing of the effector before inoculation. A similar finding has been reported in *avrPiz-t* transgenic rice plants that were more susceptible to fungal blast than was the wild-type plant (Park et al. 2012). In contrast, AvrBs2 expression in the transgenic plants did not inhibit flg22- or chitin-triggered MAPK activation, suggesting that AvrBs2 blocks immune signaling transduction downstream of MAPK in rice or inhibits other branches of defense signaling independent of MAPK cascades. Taken together, our data indicate that AvrBs2 and the identified HrpE3 and Xrp5 effectors are essential virulence factors that contribute to full virulence of *X. oryzae* pv. *oryzicola*.

In summary, the study advanced our understanding of Xop-mediated virulence in bacterial leaf streak. AvrBs2 has been demonstrated to be required for full virulence of *X. campestris* pv. *vesicatoria* on dicot plants, including pepper and tomato, through inhibition of host immunity (Zhao et al. 2011). Our results showed that AvrBs2 is also an essential virulence factor in *X. oryzae* pv. *oryzicola*, which suppresses immune responses in the monocot rice. These data suggest that AvrBs2 in *X. campestris* pv. *vesicatoria* and *X. oryzae* pv. *oryzicola* not only share high identity in protein sequence but, also, have a high function similarity. AvrBs2 might target defense signaling components shared by the dicot and monocot plants. Given the high structural and functional conservation of AvrBs2, it will be interesting to investigate whether the resistance gene *Bs2* in pepper confers disease resistance across the dicot and monocot plant classes. The finding may provide insight into strategies to exploit alien disease resistance genes in rice breeding for broad-spectrum and durable resistance to *X. oryzae*.

MATERIALS AND METHODS

Plant materials, bacterial strains, and culture conditions.

Oryza sativa L. subsp. *japonica* cvs. Jingang 30 and Nipponbare plants were grown in a greenhouse. Bacterial strains and plasmids used in this study are listed in Supplementary Table S2. A rifampicin-resistant *X. oryzae* pv. *oryzicola* RS105 was used as the wild type. The *X. oryzae* pv. *oryzicola* strains were grown in NB medium (per liter, 3 g of beef extract, 1 g of yeast extract, 5 g of tryptone, 10 g of sucrose) at 28°C. Antibiotics were used at the following concentrations (micrograms per milliliter): ampicillin, 100; kanamycin, 50; and rifampin, 25. All the experiments were repeated at least three times with similar results, unless noted.

Construction of marker-free gene-deletion mutants in *X. oryzae* pv. *oryzicola* through homologous recombination.

The predicted effector genes in *X. oryzae* pv. *oryzicola* and genome sequences were available from the *Xanthomonas* Resource and the National Center for Biotechnology Information databases (Bogdanove et al. 2011). Construction of gene-deletion mutant strains of *X. oryzae* pv. *oryzicola* was performed as described previously (Zhang et al. 2013). Briefly, genomic DNA was isolated from the *X. oryzae* pv. *oryzicola* wild-type strain RS105 using a genomic DNA isolation kit (New Industry Company, Beijing) according to the manufacturer's instructions. Two DNA fragments (approximately 1 kb) upstream and downstream close to the start and stop codons of each effector gene, respectively, were amplified from the *X. oryzae* pv. *oryzicola* genome, using the primer sets listed in Supplementary Table S3. PCR products were gel-purified and were added together into a fusion PCR reaction to amplify DNA fragment carrying upstream and downstream regions of each effector gene but lacking the open reading frame (ORF). The resultant PCR products were cloned into pUFR80 that carries a *sacB* gene as the counter-selective marker (Castañeda et al. 2005; Ried and Collmer 1987). The constructed plasmids were conjugated into *X. oryzae* pv. *oryzicola* RS105 by triparental mating. The conjugants were selected on nutrient agar (NA) solid medium plates with kanamycin selection. After culturing in NB medium without sucrose, the conjugants were plated onto sucrose (5%)-containing NA plates to screen sucrose-insensitive colonies. The gene-deletion genotypes of sucrose-insensitive *X. oryzae* pv. *oryzicola* colonies were selected using colony PCR. The mutants were subsequently subject to confirmation via Southern blot analyses.

Construction of complementation strains for *X. oryzae* pv. *oryzicola* gene-deletion mutants.

For complementation, the full-length effector genes containing upstream promoter regions (approximately 800 bp to approximately 1 kb) were amplified by PCR, using gene-specific primer sets. The amplified products were subcloned into the wide host-range vector pVSP61 (Loper and Lindow 1987). After being confirmed by sequencing, the plasmid constructs were transferred into the respective *X. oryzae* pv. *oryzicola* mutant strains by triparental conjugation. Single conjugants of *X. oryzae* pv. *oryzicola* were selected on the kanamycin-containing NA plates.

Southern blot analysis.

Southern blot analysis was performed as described previously (Cannon et al. 1979). Briefly, genomic DNA was isolated from the wild-type and mutant *X. oryzae* pv. *oryzicola* strains as described above and was then digested with appropriate restriction enzymes overnight. After separation on agarose gel, digested DNA was blotted onto Hybond-N nylon membrane (GE Healthcare, Buckinghamshire, U.K.). The probes were PCR-amplified and isotope (32 P)-labeled using the Random Primer DNA labeling kit (Takara, Dalian, China).

Transient expression of the effector proteins in *N. benthamiana*.

The coding sequence of each putative effector gene of *X. oryzae* pv. *oryzicola* without the stop codon was amplified and cloned into the vector pUC19-35S-FLAG-RBS (Li et al. 2005), after digestion with *Xho*I and *Nar*I for *avrBs2*, *Xho*I and *Csp*45I for other effector genes, except *xopAA*, *xopAB*, *xopO*, and *xopZ1*. The four effector genes were not constructed for transient expression because no suitable restriction sites are available for these genes. The ORF of each effector gene with 3×FLAG coding sequence was cleaved from the corresponding

pUC19-35S-FLAG-RBS construct with *XhoI* and *SpeI* and was religated into the DEX-inducible expression vector pTA7001 (Aoyama and Chua 1997). These constructed plasmids were transformed into the *Agrobacterium* sp. strain C58C1 through the freeze-thaw method (Deblaere et al. 1985). Overnight-cultured *Agrobacterium* strains were collected and resuspended in 10 mM MgCl₂ with 150 µM acetosyringone (optical density at 600 nm [OD₆₀₀], approximately 0.3). After incubation for 2 h or more, *Agrobacterium* cultures were infiltrated into the leaves of 6-week-old *N. benthamiana* plants with needleless syringes. These leaves were sprayed with 30 µM DEX at 24 h after infiltration. The cell-death phenotypes on the leaves were observed and photographed at 3 days after DEX spraying.

Subcellular localization and protoplast transfection assay.

For subcellular localization, the coding sequence of *GFP* was amplified from pGWB5 (Nakagawa et al. 2007) and was ligated into the pUC19 plasmid after digestion with *SphI* and *HindIII*. The 35S promoter fragment was cleaved from pUC19-35S-FLAG-RBS by *EcoRI* and *SacI* and was then religated into pUC19-GFP. The ORF of *avrBs2* was amplified using the primer set *avrBs2-BamHI-F* and *avrBs2-SalI-R* and was then cloned into pUC19-35S-GFP after digestion with *BamHI* and *SalI*. The construct was confirmed by sequencing.

The transfection assay in protoplasts was carried out as described previously (Li et al. 2005). Briefly, protoplasts were isolated from 10-day-old etiolated seedlings of *Oryza sativa* cv. Nipponbare or the leaves of 4-week-old *NHO1-LUC* transgenic *Arabidopsis* plants. The protoplasts (approximately 2.5×10^6 protoplasts per milliliter) were transfected with 10 µg of plasmid DNA by polyethylene glycol-mediated transformation. After washing with W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, and 2 mM MES-KOH, pH 5.7), the protoplasts were resuspended in W5 solution and were treated with 1 µM flg22 for 12 h, under low light. The LUC activity was then evaluated by measuring luminescence intensity 10 min after adding 50 µM luciferin to the transfected *Arabidopsis* protoplasts. GFP fluorescence on the transfected rice protoplasts was observed using confocal microscopy (Nikon Ti2000).

Development of the *avrBs2* transgenic plants and cell cultures.

The pTA7001-*avrBs2* construct was introduced into rice calli through *Agrobacterium*-mediated transformation as described previously (Ozawa 2009). Briefly, the sterilized dehusked seeds (cv. Nipponbare) were plated onto NBi medium (N6 macro elements, B5 microelements, B5 vitamin, 27.8 mg of FeSO₄·7H₂O per liter, 37.3 mg of Na₂-EDTA per liter, 500 mg of proline per liter, and 500 mg of glutamine per liter, 300 mg of casein hydrolysate per liter, 2 mg of 2,4-dichlorophenoxyacetic acid (2,4-D) per liter, 100 mg of inositol per liter, and 30 g sucrose per liter) for callus induction. The vigorous calli were picked and incubated with *Agrobacterium* cultures for 2 min and were then transferred onto NBco medium (NBi medium supplied with 100 µM acetosyringone, pH 5.5) for 3 days. The calli were subsequently cultured onto NBs medium (NBi medium supplied with 500 mg of cephamycin and 30 mg of hygromycin per liter), after washing thoroughly with sterile water. The resistant calli that emerged after approximately 3 to 4 weeks were transferred onto NBr medium (NBi medium supplied with, per liter, 0.5 mg of α-naphthalene acetic acid, 3 mg of 6-benzylaminopurine, 500 mg of cephamycin, and 30 mg of hygromycin) for shooting. The regenerated shoots were transferred onto $1/2 \times$ Murashige and Skoog medium containing 100 mg of inositol per liter for rooting. The independent transgenic plants were transferred to the greenhouse for further growth.

Rice cell-suspension cultures were initiated from the wild-type and *avrBs2* transgenic calli as described previously (Ozawa and Komamine 1989). Briefly, the compact calli with vigorous growth were inoculated in 100 ml of liquid N6 medium supplemented with 5 mg of 2,4-D and 1 mg of kinetin per liter, to establish cell suspension cultures. Hygromycin (30 mg/liter) was added for culturing the *avrBs2* transgenic calli. The suspension cultures were incubated at 26°C on a rotary shaker at 140 rpm.

Oxidative burst assays.

ROS burst in rice cell cultures was detected as described previously with minor modifications (Lu et al. 2015; Pérez and Rubio 2006). Briefly, equal volumes of calli were incubated in fresh culture medium for 24 h at 26°C. DEX was then added into cell cultures to the final concentration of 10 µM to induce *AvrBs2* expression. Ethanol (0.03%) was added into cultured cells as mock control. After incubation for another 24 h, cultured cells were washed three times and were then incubated in 3 ml of fresh media in a 20-ml vial for 3 h at 26°C. The cells were then treated with 1 µM flg22 or 20 µl of 100mg of chitin per milliliter. The supernatant (10 µl) was collected at different time points after treatments and was mixed with 1 ml of Co(II)-luminol reagent. Chemiluminescence was recorded immediately by the Infinite F200 (Tecan, Grödig, Salzburg, Austria) with the count time set as 1 s.

Protein extraction and immunoblotting.

The *avrBs2* transgenic calli treated with DEX or mock (0.03% ethanol) were collected and were ground in the centrifuge tubes with small stainless steel balls, using a milling apparatus (Retsch, Haan, Germany) for total protein extraction. The sample loading buffer (50 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 6% glycerol, 0.1 M dithiothreitol, 0.01% bromophenol blue) was added directly into the tubes. The samples were boiled for 20 min, and the supernatants were collected for gel separation. For detecting transient expression of the effectors in *N. benthamiana*, total proteins were extracted from the ground powder of infiltrated leaves, using the extraction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% protease inhibitor cocktail [Merck, San Diego, CA, U.S.A.]).

The extracted proteins were separated in a 12% polyacrylamide gel and were electrophoretically transferred onto Immobilon-P membrane (Millipore, Bedford, MA, U.S.A.) as described previously (Sun et al. 2012). Nonspecific binding sites on the membranes were blocked overnight in blocking solution consisting of 5% (wt/vol) skimmed milk in TBS-T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) at 4°C. The membranes were then incubated with an anti-FLAG monoclonal antibody (Sigma, St. Louis) at a dilution of 1:5,000 in blocking solution for 1 h at room temperature. Following three 10-min washes in TBS-T, the membranes were incubated for 1 h at room temperature in TBS-T containing horseradish peroxidase (HRP)-conjugated goat antimouse immunoglobulin G (IgG) secondary antibody (1:5,000 dilution) (CWBio, Beijing). After three washes in TBS-T, the membranes were incubated with the eECL Western Blot chemiluminescent substrate (CWBio) for 5 min and were then exposed to X-ray films. MAPK activation was detected as described (Schwessinger et al. 2011). Briefly, the immunoblots were blocked in 5% (wt/vol) bovine serum albumin (MP Biomedicals, Santa Ana, CA, U.S.A.) in TBS-T for 1 h at 4°C. Phospho-serine and threonine sites were detected using anti-Phospho-p44/42 MAPK antibody (1:2,000 dilution) (Cell Signaling Technology, Danvers, MA, U.S.A.), followed by HRP-conjugated antirabbit IgG secondary antibody (CWBio) at a dilution of 1:5,000.

RNA isolation and quantitative real-time RT-PCR.

The transgenic plant leaves and cell cultures were treated with flg22 and chitin for 6 h after treatment with DEX to induce AvrBs2 expression. Total RNA was isolated from the treated *avrBs2* transgenic plants and cells using an Ultrapure RNA isolation kit, according to the manufacturer's instructions (CWBio). Complementary DNA (cDNA) was synthesized by reverse transcription Superscript III (Invitrogen, Carlsbad, CA, U.S.A.) using total RNA as template. The transcript levels of defense genes were quantified through quantitative real time RT-PCR with SYBR premix Ex Taq (Takara) using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.). The expression level of *OsActin1* (Os03g0718100) was used as an internal reference.

Virulence assays of *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae* on rice.

Virulence of different *X. oryzae* pv. *oryzicola* gene-deletion mutant strains was determined on rice cv. Jingang 30 by pressure inoculation (Wang et al. 2007). The *avrBs2* transgenic plants were sprayed with 30 μ M DEX or 0.1% ethanol as mock control 1 day before bacterial inoculation. For bacterial leaf streak, *X. oryzae* pv. *oryzicola* strains were cultured in NB medium overnight and were diluted with sterile double-distilled water to an OD₆₀₀ of 0.3 and were then pressure-infiltrated into 6-week-old rice leaves with needleless syringes. For rice bacterial blight, the *X. oryzae* pv. *oryzae* PXO99A (OD₆₀₀, approximately 0.8) was inoculated into rice leaves by the leaf-clipping method (Kauffman et al. 1973). Disease lesions on the inoculated leaves were measured at 14 days after inoculation. At least 10 inoculated leaves were scored for each tested strain.

For establishing bacterial growth curves, leaf sections around inoculation sites were harvested at four time points (0, 4, 8, and 12 days after inoculation) and sliced into small pieces, were incubated in 5 ml of sterile water including rifampicin (25 μ g/ml) with shaking for 1 h, and then, were filtered through two layers of sterilization gauze. The filtrates were diluted and were then plated onto NA plates with rifampicin. Colonies on the plates were counted after 3 days of incubation at 28°C.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The National Center for Biotechnology Information database:
<http://www.ncbi.nlm.nih.gov>
 The Xanthomonas Resource database: <http://www.xanthomonas.org>