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Ectopic Expression of Executor Gene Xa23 Enhances Resistance to Both Bacterial and Fungal Diseases in Rice

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Abstract: Bacterial blight (BB) and bacterial leaf streak (BLS), caused by phytopathogenic bacteria Xanthomonas oryzae pv. oryzae (Xoo) and Xanthomonas oryzae pv. oryzicola (Xoc), respectively, are the most serious bacterial diseases of rice, while blast, caused by Magnaporthe oryzae (M. oryzae), is the most devastating fungal disease in rice. Generating broad-spectrum resistance to these diseases is one of the key approaches for the sustainable production of rice. Executor (E) genes are a unique type of plant resistance (R) genes, which can specifically trap transcription activator-like effectors (TALEs) of pathogens and trigger an intense defense reaction characterized by a hypersensitive response in the host. This strong resistance is a result of programmed cell death induced by the E gene expression that is only activated upon the binding of a TALE to the effector-binding element (EBE) located in the E gene promoter during the pathogen infection. Our previous studies revealed that the E gene Xa23 has the broadest and highest resistance to BB. To investigate whether the Xa23-mediated resistance is efficient against Xanthomonas oryzae pv. oryzicola (Xoc), the causal agent of BLS, we generated a new version of Xa23, designated as Xa23p1.0, to specifically trap the conserved TALEs from multiple Xoc strains. The results showed that the Xa23p1.0 confers broad resistance against both BB and BLS in rice. Moreover, our further experiment on the Xa23p1.0 transgenic plants firstly demonstrated that the E-gene-mediated defensive reaction is also effective against M. oryzae, the causal agent of the most devastating fungal disease in rice. Our current work provides a new strategy to exploit the full potential of the E-gene-mediated disease resistance in rice.

Keywords: rice; executor gene; Xanthomonas oryzae; Magnaporthe oryzae; transcription activator-like effectors

1. Introduction

Rice is a major staple food crop that feeds half of the world’s population. Bacterial blight (BB) and bacterial leaf streak (BLS), caused by Xanthomonas oryzae pv. oryzae (Xoo) and Xanthomonas oryzae pv. oryzicola (Xoc), respectively, are the most serious bacterial diseases of rice [1,2]. The two diseases, especially BB, cause severe yield losses that increase food insecurity in the world. Both Xoo and Xoc use transcription activator-like effectors (TALEs) as major virulence factors to promote rice disease susceptibility [3,4]. During the infection, TALEs are translocated into host plant cells by the bacterial type III secretion system and function as eukaryotic transcription factors in the nucleus, where they drive the expression of host susceptibility (S) genes via binding to the effector-bending element (EBE) in the promoters [5,6].

To counter the attack from TALEs, plants have evolved the so-called executor (E) genes, a unique type of resistance (R) genes identified in the plant–Xanthomonas interaction systems [7,8]. The E genes can specifically trap TALEs by using the evolved EBEs and triggering an intense
plant defense reaction characterized by a hypersensitive response (HR) [9–12]. To date, four
E genes, Xa7, Xa10, Xa2,3 and Xa27, have been cloned from rice, and they have displayed
different resistance spectrums to BB due to the different TALEs trapped [8,13]. However, no
natural E gene for BLS has been discovered, even though Xoc harbors the largest number of
TALEs in phytopathogenic bacteria [14]. It has been explained that Xoc could suppress host
resistance in multiple ways [15–17].

Despite the close relationship between Xoo and Xoc, few R genes can confer resistance
to both diseases BB and BLS [1,18,19]. The Xa1 allelic genes, which are nucleotide-binding
leucine-rich repeat (NLR)-type R genes in rice, initiate a strong resistance response by
recognizing typical TALEs in Xoo and Xoc [20–22]. Unfortunately, the broad resistance
mediated by Xa1 allelic genes was defeated by interfering TALEs (iTALEs); even some
Xa1 allelic genes, such as Xa14, can avoid the suppression of type B iTALEs [20]. To
successfully induce host susceptibility (S) genes for disease development, TALEs need to
hijack the basal transcription factor (TF) TFIIAγ5 (XA5) through the transcription factor
binding (TFB) region [23,24]. The mutation type of TFIIAγ5, xa5 encoded by the recessive
disease resistance allele of Xa5, arrests TALE-induced susceptibility by attenuating the
binding affinities of TALEs to a host TF. However, TALEs can recruit another TF, namely,
OsTFIIAγ1, to partially compensate for the attenuated susceptibility. xa5 provides race-
specific or partial resistance to Xoo and Xoc [25–27].

The TALE–EBE interaction mechanisms have been decoded by two research groups. The
repeat variable di-residues (RVDs) in every repeat in the central TALE region can specifically
recognize one base of target DNA [28,29]. Based on the RVD-EBE recognition codes, artificial EBEs
can be integrated into E genes to engineer broad and durable R genes for multiple Xanthomonas
diseases [30–32]. A single modified E gene could realize the improvement of resistance to
BB and BLS. However, Xoc is known to suppress R gene-mediated resistance [15,16,33]. Our
previous studies revealed that the E gene Xa23 has the broadest and highest resistance to BB [12].
To investigate whether the Xa23-mediated resistance is efficient for controlling BLS, here, we
generated a new version of Xa23 (designated as Xa23p1.0) by inserting a 17 bp EBE into the
Xa23 promoter (Figure 1), which can specifically trap the conserved TALEs from multiple Xoc
strains [34,35]. This work provides an alternative strategy to exploit the full potential of the
E gene-mediated disease resistance against multiple diseases in rice.

![Figure 1](image-url)  
**Figure 1.** Schematic map and nucleotide sequences of generated Xa23p1.0. A 2.5 kb fragment
containing the Xa23 locus was modified by inserting the 17 bp EBE₇al₂8 and linker sequence CCCTAT
into the promoter region via Gibson cloning.

2. Results

2.1. Generation of EBE-Modified Xa23 Transgenic Rice Plants

Our previous studies revealed that the E gene Xa23 performed the broadest resistance
against BB, because the cognate TALE (AvrXa23) conservatively exists in virtually all Xoo
strains tested [12]. However, some avirulent TALEs, e.g., AvrXa7 and AvrXa10, failed to induce the resistance in rice cultivars with the corresponding R genes after being introduced into Xoc strains [33]. To preliminarily test if the avirulent TALE can induce strong defense responses in Xa23-containing rice varieties, we transformed pHZWavrXa23 into three Xoc strains (Table S1), and the resulting engineered Xoc strains were, subsequently, inoculated into CBB23 seedlings via the needleless syringe method. For the quantitative suppression characteristics of Xoc, transformants were inoculated at different cell concentrations [16,33].

The control groups, Xoc strains carrying the empty vector, caused water-soaking symptoms in CBB23 at the maximum concentration (OD600 = 2.5) (Figure 2a). Additionally, the transformants carrying pHZWavrXa23 at different concentrations (OD600 = 0.5–2.5) all successfully developed brown spots, which is the typical symptom of HR, as a result of incompatible interactions, and bacterial spread was restricted in inoculated areas three days after infiltration (Figure 2a), implying that Xa23 can be developed into an artificial E gene against BLS. Based on the mechanism of the Xa23-mediated disease resistance, we speculated that the addition of EBE_{Tal2g}, a 17 bp nucleotide sequence recognized by the conserved TALE member Tal2g_{BLS256} from Xoc [36,37], into the promoter of Xa23 would achieve broad-spectrum resistance for both BB and BLS. To this aim, we amplified the Xa23 locus from CBB23 and generated a new E gene, designated as Xa23p1.0, by inserting the EBE_{Tal2g} upstream of the AvrXa23 binding site in the Xa23 promoter (Figure 1).

Figure 2. Generation of Xa23p1.0 transgenic rice. (a) The phenotype of CBB23 was photographed 5 days after infiltration with transformed Xoc strains containing empty vector and pHZWavrXa23 at different OD600 values. (b) Disease phenotypes of Xa23p1.0 transgenic T2 plants and wild-type Nipponbare (Nip) at tillering stage inoculated with Xoo strain PXO99A by leaf-clipping method. (c) Phenotypes of the T2 plants at mature stage in Beijing field trial.

The engineered Xa23p1.0 was, subsequently, cloned into the transformation-competent artificial chromosome vector pYLTAC380H [38] and introduced into rice variety Nipponbare (Nip) through an Agrobacterium-mediated transformation. Four (#1, #2, #3, and #4) independent Xa23p1.0-positive transgenic T0 plants were regenerated from ~1200 calli and found to be resistant to the Xoo representative strain, PXO99A (Table S1). In subsequent T1 and T2 generations, the transgenic-positive plants derived from the T0 plants retained BB
resistance (Figure 2b), but showed a somewhat stunted growth, characterized by a shorter plant height and less tillers (Figure 2c). We examined the T2 plants using q-PCR and found that the Xa23p1.0 transgenic plants had differential levels of constitutive Xa23 expression (Figure 3a). This was consistent with the speculation that the leaky expression of an E gene could cause stress-related phenotypes in transgenic plants [9,32].

Figure 2. Generation of Xa23p1.0 transgenic rice. (a) ... resistance suppression using Xoc was quantitative [16,33]. Here, RS105, the representative Xoc strain, was firstly

When the corresponding avirulence (avr) gene, avrXa23, was expressed from a high-copy plasmid, we observed a strong HR in CBB23 (Figure 2a). However, we still needed to firstly investigate whether the Xa23-mediated resistance induced by the endogenous avr gene on the chromosome was efficient for BLS. For that, the resistance suppression using Xoc was quantitative [16,33]. Here, RS105, the representative Xoc strain, was firstly used to verify the resistance of Xa23p1.0 in the positive T2 plants derived from the four transgenic rice lines, via needleless syringe infiltration (Figure 3b). The BLS lesions in the T2 plants of Xa23p1.0-transgenic rice were obviously shorter than that in the control Nipponbare. However, the inoculated spots did not turn brown in the transgenic rice plants, which was different from spots caused by RS105/pHZWavrXa23 in CBB23. The results revealed that suppression of Xa23-mediated resistance existed in rice-Xoc interactions. In addition, the water-soaking symptoms caused by RS105 in the transgenic lines #1 and #2 seemed stronger than that in line #3 and #4 (Figure 3b), demonstrating a high background expression that seemed to confer a strong resistance in rice against Xoc. It also hinted that Xoc produced a dose-dependent suppression of the Xa23-mediated disease resistance response.

To accurately evaluate Xa23p1.0 resistance to BLS and test the effectiveness of combining EBE_{Tal2g} and Xa23, five highly virulent Xoc strains (Table S1), together with RS105 and BLS256, were used to inoculate the T2 plants derived from the T0 plant #1, whose basal level of expression of xa23/Xa23 was similar to that in Nipponbare. Disease assay results showed that the T2 plants were highly resistant to all the Xoc strains, characterized by significantly shorter lesions (Figure 4a,b). Consistent with the resistance phenotype, the Xa23 expression was significantly induced in the T2 plants when challenged with Xoc (Figure 4c). The population growth of RS105 was significantly reduced in the #1 plant relative to the control Nipponbare (Figure 4d). These results indicated that EBE_{Tal2g} in the Xa23p1.0 promoter successfully trapped the conserved TALEs from Xoc. In short, the above data clearly demonstrated that Xa23p1.0 conferred broad resistance to both BB and BLS in rice.
used to verify the resistance of Xa23p1.0 in the positive T2 plants. We performed pathogenicity assays using an leaf punch-inoculated on detached leaves from the abovementioned T2 plants. 

Significant difference between values (t-test, *p < 0.05). The expression of Xa23 was evaluated by qRT-PCR at 48 h post inoculation with Xoc strain RS105. Data are means ± SD of four biological replicates. 

Bacterial populations in the leaves challenged with Xoc strain RS105. Data are means ± SD of four biological replicates. 

Bacterial populations in the leaves challenged with Xoc strain RS105. Data are means ± SD of four biological replicates. 

Bacterial populations in the leaves challenged with Xoc strain RS105. Data are means ± SD of four biological replicates. 

The defense pathway against fungal pathogens may overlap with the defense pathway against bacterial pathogens in rice. Additionally, no studies have confirmed that the E-gene-mediated disease resistance is effective against fungal pathogens. Blast caused by the fungus Magnaporthe oryzae is another destructive disease of rice. To inspect whether E gene expression is valuable for resistance against fungal pathogens, we conducted blast resistance assays on the Xa23p1.0 transgenic plants. Therefore, Rb-17, a virulent M. oryzae isolate (Table S1), was punch-inoculated on detached leaves from the abovementioned T2 plants at the seedling stage. The blast lesions caused by Rb-17 were obviously shorter in the transgenic plants compared with the wild type (Figure 4e,f). It is noteworthy that the transgenic lines derived from T0 plants #2, #3, and #4 with a higher leaky expression of Xa23 conferred a stronger resistance to M. oryzae (Figure 4e,f). These results suggested that Xa23-mediated disease resistance could also be effective against rice blast. Meanwhile, we found that the gene expression level correlated with the intensity of the BLS and blast resistance.

2.3. The Stressed Phenotypes of Xa23p1.0 Transgenic Plants Were Relieved in the Sanya Field Trial

As mentioned previously, the Xa23p1.0 transgenic rice plants exhibited stress phenotypes and yield loss due to the leaky expression of Xa23. The leaky expression of the E gene led to an over-response of the immune system in plants. To investigate the resistance intensity of stressed plants, we performed pathogenicity assays using an avrXa23-deleted mutant, PΔavrXa23, through leaf-clip inoculation in T2 plants derived from the transgenic line #3, which had the highest leaky expression level of Xa23p1.0 based on the q-PCR results. PΔavrXa23 is an avrXa23 deletion mutant derived from PXO99A, and has high virulence in Xa23-containing rice. PΔavrXa23 and the wild type caused similar lesion lengths in the susceptible control Nipponbare, while the T2 plants of #3 exhibited strong resistance to both the virulent and avirulent Xoo strains (Figure 5a). These results indicated that the activated immunity in the #3 plants could guard hosts from multiple pathogen infections.
Although the \textit{Xa23p1.0} transgenic rice successfully acquired a stronger disease resistance, over-reactive immune responses could induce a negative impact on agronomic characters. To evaluate the influence for the related agronomic characters, we conducted two independent field trials of T\textsubscript{2} plants from #3 and the wild-type Nipponbare in Beijing (BJ) and Sanya (SY) in 2021. Most obviously, the plant height, number of tillers, number of panicles, and seed weight per plant were drastically decreased for excessive immune response in the two field trials. The main environmental difference between our two experimental stations was the monthly average temperature. The temperature of Sanya station was slightly higher than that in Beijing, and the total growth period of the rice plant is shorter in high temperatures. Additionally, the stressed phenotypes were more remarkable in the Beijing field trial in the summer (June through October). The T\textsubscript{2} plants from #3 only had 33.3\% tillers and 31\% panicles in comparison to the wild-type Nipponbare (Figure 2c and Table 1). A reduction in tiller numbers is the major cause for serious yield losses (about 53.1\%) per plant, and the plant height and seed setting rate also significantly decreased. However, in the field trial in a warm climate (Figure 5b), the phenotypic differences between the #3 T\textsubscript{2} plants and Nipponbare were narrowed down. The number of panicles and seed weight per plant of the transgenic rice were recovered to 73.8\% and 77.7\% of the wild type, respectively. Particularly, the plant height of the transgenic rice was close to that of the wild type. These findings indicated that the stressed phenotypes of the \textit{Xa23p1.0} transgenic line, #3, were significantly relieved in the field trial in the tropical region. In addition, although the transgenic rice exhibited intense stressed phenotypes, the thousand seed weight was not obviously different between the #3 T\textsubscript{2} plant and Nipponbare. We also found that the seed setting rate of the #3 T\textsubscript{2} plant and Nipponbare decreased sharply in the Sanya field trial.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Leaky expression of \textit{Xa23} led to over-response of the immune system. (a) Disease phenotypes of T\textsubscript{2} plants of line #3 and wild-type Nipponbare (Nip) at tillering stage inoculated with PXO99\textsuperscript{A} and \textit{avrXa23} deletion mutant P\textit{ΔavrXa23} by leaf-clipping method at OD600 = 1.0. (b) Phenotypes of the T\textsubscript{2} plants of line #3 at mature stage in Sanya field trial.}
\end{figure}
3. Discussion

Unlike the nucleotide-binding leucine-rich repeat (NLR)-type R genes, which are common and abundant [39], a few E genes (only six cloned to date) consist of a unique type of plant R genes. These E genes are TALE-dependent, and they can specifically trap the TALEs of pathogens and trigger an intense defense reaction characterized by HR in the host. This strong resistance is a result of programed cell death induced by the E gene expression that is only activated upon the binding of a TALE to the EBE located in the E gene promoter during a pathogen infection [2]. According to the TALE–DNA binding principle, artificial EBE sequences can be integrated into promoters of E genes to generate broad-spectrum resistance by trapping expected TALEs. However, the E genes are often expressed constitutively using the conventional transgenic approach.

CRISPR-Cas9-mediated precise homology-directed repair (HdR) provides an ideal strategy to modify the promoters of E genes and their alleles at a native locus for preventing leaky expression [40]. The other notable thing is that endogenous E gene (or the allele) loci are absent in some rice varieties; for example, Nipponbare does not contain Xa27 and its allele [41]. The exploration of more valuable members is important for E gene utilization in biological breeding for resistance to BB and BLS. It has been reported that the insertion of EBEs into the Xa27 promoter conferred sufficiently strong resistance to Xoo and Xoc in rice [30]. However, the suppression of host resistance was observed in multiple plant–pathogen interaction systems, such as the rice–Xoc interaction system. To evaluate the prospects of applying Xa23 in controlling BLS, we developed an EBE-amended Xa23, designated as Xa23p1.0. Afterwards, Xa23p1.0 was integrated with EBE sequences targeting conserved TALEs in both Xoo and Xoc, and conferred broad-spectrum resistance to two bacterial diseases in rice. The results also indicated that Xoc could not successfully suppress Xa23-mediated resistance in rice.

HR-like cell death is the most effective plant immune response restricting pathogen invasion and growth [42,43]. The E-gene-mediated HR has often been accompanied by typical brown spots at the inoculation sites in rice. When the wild Xoc isolate was injected into the Xa23p1.0 transgenic rice plants, the plant leaves exhibited resistance against pathogens, but the inoculated spots did not appear to like what was induced by AvrXa23 expressed from a high-copy plasmid in Xoc (Figure 2a). Simultaneously, we found that the BLS symptoms were less severe in the Xa23p1.0 higher leaky expression plant (Figures 3b and 4a). These observations reinforced the view that rice resistance to BLS is quantitative, and the suppression of host innate immunity generally exists in Xoc–rice interactions. The results hinted that a high-level expression of Xa23p1.0 could defeat the immunosuppressive effects of pathogens. To acquire strong resistance for BLS, multiple tandem EBEs can be integrated into promoters to trap more TALE members to induce a high-level expression of the E gene.

Meanwhile, the Xa23p1.0 transgenic rice provided opportunities to test the Xa23-mediated resistance to other pathogens in rice. Virulence assays on rice seedlings indicated that the leaky expression of Xa23p1.0 enhanced host resistance to M. oryzae. The over-activation of immune response conferred a strong host resistance to multiple pathogens without a specific recognition mechanism in the E-gene-mediated resistance. However,

### Table 1. Agronomic characteristics of Nipponbare and Xa23p1.0 transgenic rice in Beijing and Sanya field trials.

| Rice Lines | Plant Height (cm) | Number of Tillers | Number of Panicles | Panicle Length (cm) | Seed Setting Rate (%) | Thousand Seed Weight (g) | Seed Weight per Plant (g) |
|------------|------------------|-------------------|--------------------|---------------------|------------------------|--------------------------|---------------------------|
| BJ Nip     | 67.88 ± 4.91     | 22.50 ± 1.86      | 21.00 ± 1.80       | 17.34 ± 0.95        | 79.45 ± 2.36           | 25.33 ± 0.87             | 26.21 ± 0.95              |
| #3         | 52.75 ± 2.45     | 7.50 ± 0.87       | 6.50 ± 1.22        | 12.93 ± 1.17        | 58.36 ± 4.27           | 26.25 ± 0.48             | 12.28 ± 2.19              |
| SY Nip     | 64.80 ± 1.55     | 26.30 ± 1.73      | 25.20 ± 2.33       | 16.40 ± 0.81        | 69.69 ± 3.99           | 27.37 ± 0.92             | 25.80 ± 3.13              |
| #3         | 59.00 ± 2.45     | 19.50 ± 2.06      | 18.60 ± 2.41       | 16.05 ± 0.56        | 68.87 ± 6.93           | 26.57 ± 1.01             | 20.05 ± 1.54              |

Results are shown by average data (the mean ± SD) from ten plants of each line. Different letters indicate a statistically significant difference between values (p < 0.05). Beijing, BJ; Sanya, SY.
the leaky expression of Xa23p1.0 stressed the phenotypes in transgenic rice, leading to an over-response of the immune system. Notably, based on the agronomic data in Beijing and Sanya, we found that the stressed phenotypes of Xa23p1.0 transgenic rice were relieved in the field trial in the tropical region. It was reported that Xa7 conferred a stronger rice resistance at higher temperatures. There were differences in multiple climatic factors between Beijing and Sanya. Does that imply that plants have some regulatory mechanisms to adjust the negative influence of an immune response under high temperatures? In the future, we would like to study the effect of temperature in the E-gene-mediated resistance.

E genes can achieve broad resistance through integrating pathogen-responsive cis-elements, such as EBEs, into the promoters of E genes or their alleles to sense multiple pathogen effectors. The E-gene-mediated immune response can protect the host against bacterial and fungal pathogens. CRISPR-Cas9-mediated precise homology-directed repair might be an ideal strategy to create resistant germplasms, because some unknown elements should exist at a native locus to control the background expression of E genes. The low basic levels of expression of E genes avoid the damage caused by the over-response of the immune system. In conclusion, our work successfully expanded the Xa23 resistance spectrum from BB to BLS by adding EBE\textsubscript{tal2g} in the promoter region. Furthermore, we demonstrated that the Xa23-mediated defensive reaction was also effective against fungal M. oryzae, the causal agent of rice blast. These findings imply that the identification of pathogen-responsive cis elements with a low background expression and efficient pathogen induction activity might be a key factor for exploiting the full potential of E gene resistance in the future.

4. Materials and Methods

4.1. Plant Materials and Bacterial Strains

For seedling inoculation and field trail, seeds of Nipponbare and Xa23p1.0 transgenic plants were germinated in Petri dish for 5 days in a 30 °C dark incubator. Seedings were transplanted to a plastic box (61 × 42 × 15 cm) with Pindstrup substrate (P8988, Ryomgaard, Denmark). Plants were grown in a greenhouse at 26 to 32 °C with a light and dark cycle of 14 and 10h, until they were at five-leaf stage. Escherichia coli strains were grown in Luria-Bertani (LB) medium supplemented with appropriate antibiotics at 37 °C. Agrobacterium tumefaciens strains were grown at 30 °C under the dark. All Xoo and Xoc strains were grown at 28 °C in nutrient broth (NB) (1% peptone, 0.5% yeast extract, 1% sucrose, and 1.5% agar). Antibiotics (Sangon, Shanghai, China) were used at the following concentrations if required: 100 µg/mL ampicillin, 25 µg/mL rifampicin, 25 µg/mL kanamycin, and 100 µg/mL spectinomycin.

4.2. Field Experiment

To evaluate the agronomic characteristics under normal field conditions, both Nipponbare and Xa23p1.0 transgenic rice were grown to five-leaf stage in greenhouse (see Section 4.1). Then, the seedings were planted in a three-row plot with ten plants per row, applying 20 × 16 cm spacing at Hainan experimental station (18.396448 N, 109.203067 E) of Institute of Crop Sciences from January to May and Shunyi experimental station (40.237433 N, 116.570354 E) of Institute of Crop Sciences from June to October and January to May in 2021. Ten plants in the middle row of each line were sampled for the agronomic characteristics investigation.

4.3. Plant Inoculations and Disease Assays

For isolating new Xoc strains, rice leaves with typical BLS symptoms were firstly collected from paddy in southern China from 2018 to 2021. After surface sterilization, the leaves were crushed in microcentrifuge tubes containing 500 µL distilled H\textsubscript{2}O using a plastic pestle. Then, the bacterial suspension was serially diluted and plated onto NA plates incubated at 28 °C for 4 days. The suspected Xoc clones were selected, purified, and identified using specific primers with colony PCR amplification (Table S2) [14].
For *Xoo* and *Xoc* inoculation, bacterial cells were collected from culture at low-speed (4000 r.p.m.) centrifugation, washed twice and suspended in sterile water. The suspensions were adjusted to required optical density, e.g., OD600 = 1.0 (approx. 1 × 10⁹ cell/mL), as described previously [21]. The adult rice plants were inoculated in *Xoo* suspension by the leaf-clipping method in the fields of our experimental station and *Xoc* suspension by needleless syringe infiltration method in greenhouse and pin-pricking method in fields of our experimental station. Lesions were photographed and measured 15 days after inoculation (DAI) for leaf-clipping and pin-pricking inoculation. For syringe infiltration inoculation, lesions were photographed and measured 5 DAI.

To measure bacterial populations, infiltrated areas of rice leaves were sterilized and crushed as above. Then, samples were diluted serially in sterile water and spread onto NA plates. Plates were inoculated at 28 °C until single colonies could be counted. Results were displayed as the mean and standard deviation of all measurements for three replicates. For *M. oryzae* inoculation, the punch inoculation method was conducted as described previously [44]. Briefly, isolate Rb-17 was cultured on oat meal agar medium for 14 days for generating spores. Spores were collected by washing the agar cultures with sterile water (containing 1% Tween 20), and the spore concentration was adjusted to ~5 × 10⁵ spore/mL. Detached seedling leaves were wounded with a hole-punch. An amount of 7 µL spore suspension was applied to the injured area, and then the inoculated leaves were placed in sterile water that contained 0.1% 6-Benzylaminopurine to keep them moist. The inoculated leaves were kept in darkness at 28 °C for 24 h before they were transferred to a controlled growth chamber. Lesions were photographed and measured when significant difference was observed.

The disease assays were performed at least twice. Three replicates with approximately 3 leaves from three to five plants per replicate were inoculated per strain. One-way analysis of variance (ANOVA) statistical analysis was conducted on all measurements. Tukey’s honestly significant difference test was used for post-ANOVA pair-wise tests for significance, set at 5% (*p* < 0.05).

### 4.4. Genes and Constructs

The upstream (~1299 bp) and downstream (~940 bp) fragments of *Xa23* were amplified from CBB23 gDNA, and the 17 bp nucleotide sequence of EBE<sub>Tal2g</sub> was inserted into the native promoter of *Xa23* through Gibson assembly method (C113-01, Vazyme, Nanjing, China) to generate *Xa23p1.0* (Table S2). Then, *Xa23p1.0* was ligated into pYLTAC380H and introduced into Nipponbare using *Agrobacterium*-mediated transformation described previously [12].

### 4.5. qRT-PCR Analyses

To analyze the gene induction, three-week-old rice seedlings were selected for *Xo* (OD600 = 1.0) inoculation with the needleless syringe method. The treated and untreated leaves were harvested and grinded in Trizol (Thermofisher, Waltham, USA) to extract total RNAs. Then, the treated RNA samples were reversely transcribed into cDNA by reverse transcription Kit (TIANGEN, Beijing, China). qRT-PCR was performance on ABI 7500/7500 Fast Real-Time PCR system using SYBR<sup>®</sup> Premix ExTaqTM II kit (TaKaRa, Dalian, China). The reaction volume was 20 µL, containing 10 µL 2 × SYBR Green Master Mix, 2 µL cDNA, 0.4 µL 50 × ROX Reference Dye II, 0.8 µL forward and reverse primers (10 µmol L⁻¹), and 6 µL RNase-free H₂O. The PCR procedure was predenaturation at 95 °C for 2 min, 95 °C for 15 s, 60 °C for 30 s, and 40 cycles. The rice Actin gene was set as internal reference gene (Table S2). The 2<sup>−ΔΔCt</sup> method was used as a relative quantification strategy for data analysis, and three biological replicates were measured for each sample.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23126545/s1 [45].
Author Contributions: Z.J. and K.Z. designed the experiments. Z.J., H.S., Y.W., M.L. and H.W. performed the experiments. J.X. and C.L. conducted the pathogen inoculation. Z.J., C.W. and K.Z. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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