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Identification and Expression Pattern Analysis of Bacterial Blight Resistance Genes in *Oryza officinalis* Wall ex Watt Under *Xanthomonas oryzae* Pv. *oryzae* Stress

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Abstract

Background Bacterial blight (BB) caused by *Xanthomonas oryzae* Pv. *oryzae* (*Xoo*) is one of the most serious diseases of rice worldwide. *Oryza officinalis* Wall ex Watt, harboring abundant genetic diversity and disease resistance features, are important resources of exploring resistance genes with broad-spectrum resistance to BB. However, the molecular mechanisms and genes of BB resistance in *O. officinalis* have been rarely explored.

Results Here, the BB resistance of four different origin *O. officinalis* populations in Yunnan were identified by seven representative hypervirulent *Xoo* races, which exhibited different BB resistance among four populations, in which the BB resistance of the Gengma_Lincang population was the strongest. In addition, the pathogenetic ability of seven *Xoo* races to *O. officinalis* was different in that the pathogenicity of PXO99 was stronger than that of C5. There were no remarkable differences in leaf microstructures among four *O. officinalis* populations, revealing the differences in resistance of four *O. officinalis* to BB are caused by the endogenous resistance genes. Furthermore, our results proved that there were no nine cloned BB resistance genes in four populations but possessed dominant *Xa5*, dominant *Xa13*, and recessive *xa3/xa26* homologous alleles of *xa5*, *xa13*, and *Xa3/Xa26* resistance genes. These three homologous genes were isolated and cloned from four populations and named *OoXa5*, *OoXa13*, and *Ooxa3/xa26*. The expression profile revealed that the expression levels of *OoXa13* and *Ooxa3/xa26* were significantly down-regulated under PXO99 and C5 stress, especially in the Gengma_Lincang population, suggesting the *O. officinalis* might enhance BB resistance by down-regulating the expression level of *OoXa13* and *Ooxa3/xa26*.

Conclusion The BB resistance genes of *O. officinalis* had its own characteristics by expression pattern and BLAST analysis of *OoXa5*, *OoXa13*, and *Ooxa3/xa26*, which indicated that there might be new genes or molecular mechanism of BB resistance in *O. officinalis*. Our studies provided a solid foundation and reference for revealing the molecular mechanism of BB resistance in *O. officinalis*.

Keywords *Oryza officinalis* Wall ex Watt · Bacterial blight · Leaf microstructure · Resistance genes · Gene expression

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Introduction

Rice is the main cereal crop for half of the world's population, while rice bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is the fourth serious bacterial disease in the world (Mansfieldi et al. 2012; Rasabandith 1998). BB has been reported in all major rice-growing regions of the world (Nino-Liu et al. 2006). Nowadays, the most economical and effective strategy to control BB is cultivating resistant cultivars, but the number of resistance genes identified from

the cultivated rice is very limited, and more cultivars with narrow-spectrum BB resistance genes become susceptible to diseases with the emergence of new physiological races. Therefore, it is very important to identify more resistance resources and to explore new resistance genes for breeding resistant cultivars with broad-spectrum resistance to BB.

Wild rice species, the ancestor of cultivated rice, is an important gene pool harboring lots of good characteristics, such as strong resistances to blight disease, blast, insects, drought, and coldness, and the resistance genes which can be used to improve the genetics and agronomic traits of the cultivated rice (Fan et al. 2000; Fan 2000; Qin et al. 2000). The number of wild rice species in the Yunnan Province is the largest among all provinces in China (Cheng et al. 2004; Zhang 2002; Gao et al. 2000). *Oryza officinalis* is resistant or tolerant to many common rice diseases. Zhang et al. (1994) found that 50% of the highly resistant materials are *O. officinalis* among the Chinese wild rice species. The Gengma_Yunnan *O. officinalis* population has high resistance to BB and brown planthopper and medium resistance to rice blast (Peng et al. 1982). There are several *O. officinalis* populations in Yunnan, and these populations have some differences in leaf size, plant height, and width. However, the BB resistance levels and the resistance genes of *O. officinalis* populations remain unclear. Meanwhile, there were many physiological races of *Xoo* in which their pathogenetic ability to Yunnan *O. officinalis* was unclear. So far, there is lack of knowledge about systematic identification of Yunnan *O. officinalis* populations against *Xoo* races.

Previous studies have identified 42 rice BB resistance genes (Vikal and Bhatia 2017), but only nine genes have been cloned, including *Xa1* (Yoshimura et al. 1998), *Xa3/Xa26*, *xa5* (Iyer-Pascuzzi and McCouch 2004), *Xa10* (Gu et al. 2008), *xa13* (Chu et al. 2006a; Sanchez et al. 1999), *Xa21* (Song et al. 1995; Ronald et al. 1992), *Xa23* (Fan et al. 2011; Zhang et al. 1998), *xa25* (Lee et al. 2003), and *Xa27* (Gu et al. 2004). The identification and cloning of BB resistance genes provide gene resources for resistance breeding of rice. However, due to the race specific genes and the evolution of new *Xoo* races, the existing BB resistance genes are severely restricted in the application of resistance breeding (Bhasin et al. 2012; Kameswara et al. 2002). *O. officinalis* has high resistance to BB and may possess new BB resistance genes. Seven reported and cloned BB resistance genes, including *Xa1*, *Xa3/Xa26*, *xa5*, *xa13*, *Xa21*, *Xa23*, and *Xa27*, were detected in *O. officinalis* using molecular markers and functional markers tightly linked to them (Li et al. 2015). However, it is not clear if these BB resistance genes exist in all the Yunnan *O. officinalis* populations. What is more, the functional domain and resistance level of BB resistance genes between *O. officinalis* and cultivated rice have not been reported.

In this study, seven representative hypervirulent *Xoo* races were used to systematically identify the resistance of four

O. officinalis populations, Gengma_Lincang, Menghai_Xishuangbanna, Jingne_Jinghong, and Lancang_Puer, of the Yunnan Province. Meanwhile, the molecular markers linked to nine cloned BB resistance genes were used to detect *O. officinalis* to confirm whether these genes exist in the four populations. The detected *Xa5*, *Xa13*, and *xa3/xa26* homologous genes were also cloned from the four populations. Expression patterns of the three homologous genes under *Xoo* stress were analyzed by qRT-PCR. In addition, the leaf microstructures, relating to the propagation and spreading velocity of *Xoo*, of four *O. officinalis* populations were observed by microscopy. Our studies provided a solid foundation and reference for revealing the molecular mechanism of BB resistance in *O. officinalis*.

Materials and Methods

Plant Materials and Hypervirulent *Xoo* Races

Oryza officinalis Wall ex Watt populations were collected from four places of Yunnan Province, viz., Gengma_Lincang, Menghai_Xishuangbanna, Jingne_Jinghong, and Lancang_Puer, and planted in a greenhouse. The materials of IRBB1(*Xa1*), IRBB5 (*xa5*), IRBB10 (*Xa10*), IRBB13 (*xa13*), *Oryza longistaminata* (*Xa21*), *Oryza rufipogon* (*Xa23* and *Xa27*), Minghui63 (*Xa25* and Xa3/ *Xa26*), IR24 (*Xa5*), IR64 (*Xa13*), and 02428 *japonica* were also planted in a greenhouse. Seven hypervirulent *Xoo* races (Table S1) were used to screen the four *O. officinalis* populations for identification of BB resistance.

Identification of BB Resistance Among Four *O. officinalis* Populations

The seven *Xoo* races were cultured on nutrient agar medium at 28 °C for 48 h. The culture was suspended in sterile water, and the concentration was adjusted to $OD_{600} = 0.8$–1.0 using the NanoDrop2000 and, then, used to inoculate four *O. officinalis* populations along with the susceptible *Oryza sativa* cultivar 02428 at the booting stage by leaf-clipping method (Kauffman et al. 1973) at 14:00–15:00 time under 28–30 °C room temperature. At least three plants were inoculated by each *Xoo* race, and each leaf was cut 1–2 cm from the tip of the leaf.

The lesion length and lesion area were measured and calculated after inoculation with *Xoo* for 21 d. The average of the three plants was taken as the resistance index, and lesion length and lesion area were used to classify resistance or susceptibility by the BB national standard classification (Waheed et al. 2009) (Table S2). Meanwhile, the pathogenetic ability to four *O. officinalis* populations of the seven *Xoo* races was analyzed according to the leaf lesion area.

The Microstructure Observation of Four *O. officinalis* Populations

The middle part of the third fully expanded leaf was selected to crosscut by free-hand section. The thin slices were observed and photographed by inverted fluorescence microscope (Leica DMI4000B). The macro-vascular bundle area, air cavity area, and leaf thickness were measured by the data analysis software of Leica. The numbers of macro-vascular bundle, small-vascular bundle, and air cavity of the leaf midveins were also counted.

The PCR Identification of Nine Cloned BB Resistance Genes in Four *O. officinalis* Populations

Nine cloned BB resistance genes, including *Xa1*, *xa5*, *xa13*, *Xa21*, *Xa23*, *Xa26*, *Xa27*, *Xa10*, and *xa25*, were detected in four *O. officinalis* populations. In the identification of *Xa23* gene in *O. officinalis*, the EST molecular marker C189, which was tightly linked to it, was utilized (Li et al. 2015). The *Xa21* and *Xa27* genes were identified based on the functional markers designated by the polymorphic loci of their sequences (Li et al. 2015; Fan et al. 2011). In the amplification of the other six cloned BB genes, the specific primers designated by their conserved sequences were utilized (Table S3). Meanwhile, two primers provided by Hur et al. (2013) were used to identify the *Xa3/Xa26* or *xa3/xa26* in *O. officinalis*. The genomic DNA of all the materials was extracted from fresh leaves followed by the CTAB method (Murray and Thompson 1980). The 50 μL PCR reaction system contained 1 μL DNA template (50 ng L^{-1}), 2 μL each primer (10 μmol L^{-1}), 25 μL 2 × Power Pfu PCR MasterMix (TaKaRa, Dalian, China), and 20 μL ddH$_2$O. The PCR amplification program was as follows: pre-denaturation at 94 °C for 2 min; 94 °C for 15 s, 52–65 °C for 15 s, and 72 °C for 30 s–1 min, at 30 cycles; and 72 °C for 2 min. The PCR products were separated by the 2–4% agarose gel electrophoresis.

Isolation and Cloning of *Xa5*, *Xa13*, and *xa3/xa26* Homologous Genes from Four *O. officinalis* Populations

In order to clone the *Xa5*, *Xa13*, and *xa3/xa26* homologous genes from the four populations, the primers were designated by Primer 5.0 using their ORF sequences (Table S4). Total RNA was extracted from the four populations following the plant RNA kit (Omega Bio-Tek, Georgia, USA), and the first-strand cDNAs were produced by retranscription of total RNA with PrimeScript™ RT reagent (TaKaRa). The cDNA template of the four populations and PrimeSTAR®Max DNA Polymerase (TaKaRa) were used to establish 50 μL PCR reaction system to clone *OoXa5*, *OoXa13*, and *Ooxa3/xa26*.

The PCR amplification program was as follows: pre-denaturation at 98 °C for 2 min; 98 °C for 10 s, 50–55 °C for 5–15 s, 72 °C for 5–15 s, at 35 cycles. The PCR products were separated by the 2–4% agarose gel electrophoresis, and, then, the products with the expected sizes were isolated by gel extraction kit (TaKaRa) and cloned into pClone007 vector (TSINGKE) to sequence.

BLAST Analysis of *OoXa5*, *OoXa13*, and *Ooxa3/xa26*

The ORF of *OoXa5*, *OoXa13*, and *Ooxa3/xa26* was searched through the ORF online software. The three fragments of BF26, SE26, and LA26 were spliced by DNAMAN to obtain the complete ORF of *Ooxa3/xa26*. The sequences of *Xa5/xa5* (Nipponbare and IR24/IRBB5), *Xa13/xa13* (IR24 and IR64/IRBB13), and *xa3/xa26/Xa3/Xa26* (IR24/Minghui63 and IRBB3) were downloaded from NCBI. The nucleotides and amino acids of *OoXa5*, *OoXa13*, and *Ooxa3/xa26* were aligned with *Xa5/xa5*, *Xa13/xa13*, and *xa3/xa26/Xa3/Xa26*, respectively, using the DNAMAN. The functional domains of *Ooxa3/xa26* from the four populations and *xa3/xa26/Xa3/Xa26* were predicted by an online tool (http://smart.embl-heidelberg.de/).

Expression Pattern Analysis of *OoXa5*, *OoXa13*, and *Ooxa3/xa26* in Response to *Xoo*

Total RNA was extracted from the leaves after inoculation with ddH$_2$O, PXO99, and C5 for 0, 24, 48, 72, 96, and 120 h according to the manufacturer's instructions of the plant RNA kit (Omega Bio-Tek). A total of 1 μg RNA was reverse-transcribed into cDNA using PrimeScript™ RT reagent with the gDNA Eraser kit (TaKaRa). The quantitative real-time polymerase chain reaction (qRT-PCR) primers of *OoXa5*, *OoXa13*, and *Ooxa3/xa26* were designed by an online tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast) based on their ORF sequences (Table S5). Gene expression levels were determined by performing qRT-PCR in Applied Biosystems QuantStudio 6 Flex (ABI, USA) using SYBR Premix Ex Taq II (TaKaRa) according to the manufacturer's instructions. Data were analyzed by QuantStudio 6 Flex software (ABI, USA) and the $2^{-\triangle\triangle CT}$ method (Bustamante et al. 2014; Livak and Schmittgen 2001).

Results

The Identification of BB Resistance Among Four Yunnan *O. officinalis* Populations

The BB resistance of four *O. officinalis* populations was different by testing the lesion area, among which the Gengma_Lincang population had the strongest resistance (Figs. 1a and 2). The BB resistance level of the

Gengma_Lincang population was HR or R, while the resistance level of the other three populations was similar from MR to R (Figs. 1a–d, 2, and Table S6). Although the resistance level of the four populations against seven *Xoo* races was different, all of them had strong resistance to BB. The characteristics of high resistance to BB were particularly outstanding in the Gengma_Lincang population, which showed high resistance to multiple *Xoo* races (BD8438, T7147, C9, and C5),

suggesting the Gengma_Lincang population might have richer BB resistance genes than the other three populations or the Gengma_Lincang population had a BB resistance gene family which might confer resistance specific to each *Xoo* race.

Seven *Xoo* races had different pathogenicity to *O. officinalis*. The resistance reaction of the same *O. officinalis* population showed obvious difference to different *Xoo* races. The lesion length and lesion area of the four

Fig. 1 Bacterial blight disease reaction of four different *O. officinalis* populations and cultivar 02428 against different *Xoo* races. All the materials were inoculated with seven *Xoo* races at the booting stage, and the top second leaves were chosen for inoculation. The lesion length and lesion area were analyzed at the 21st day after inoculation with *Xoo* races
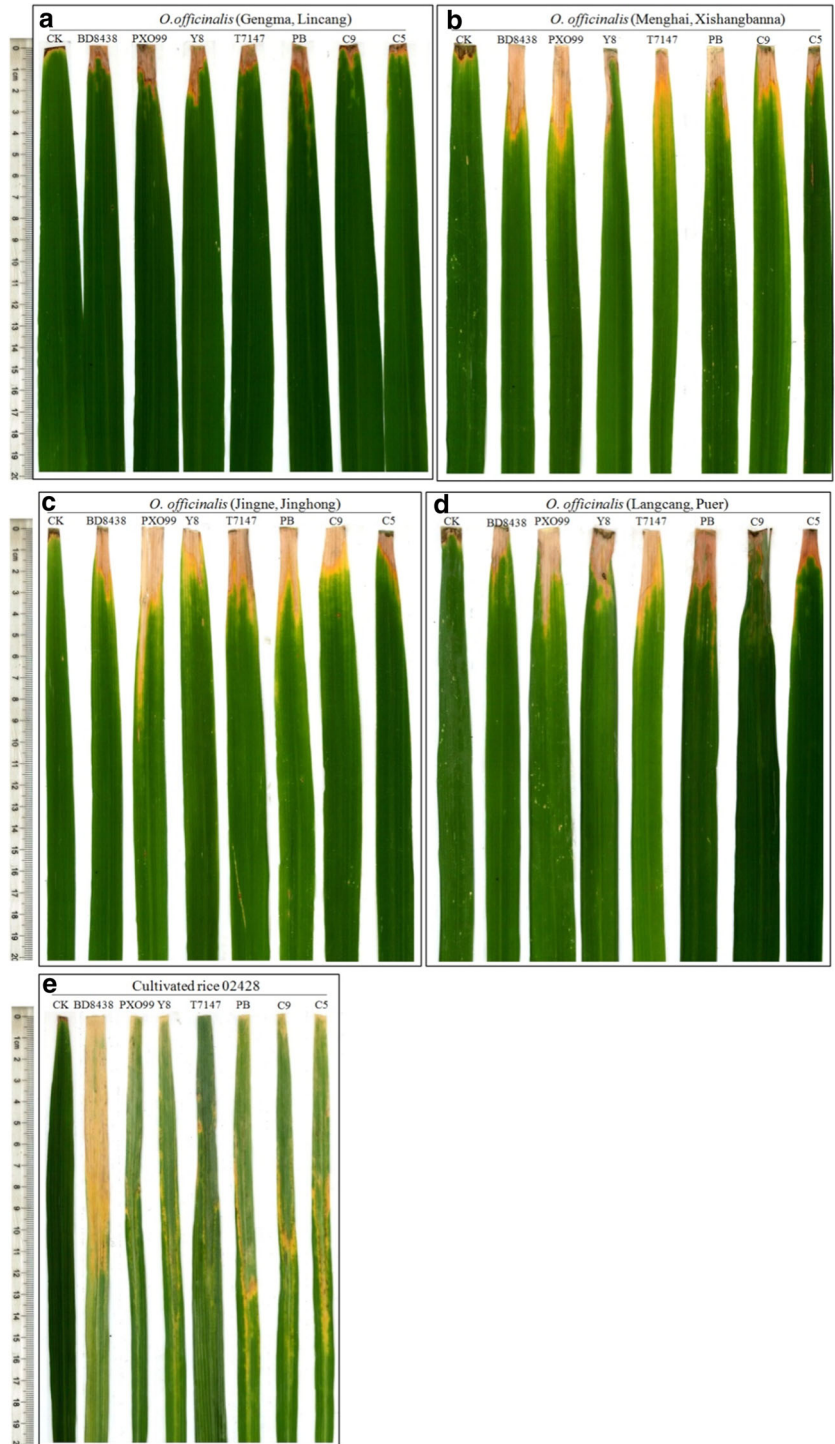
populations inoculated with PXO99 were the longest and largest, whereas the lesion length of the four populations inoculated with C5 was the shortest (Fig. 2), especially more than 90% of the Gengma_Lincang population showed high resistance (HR) to C5. The lesion almost extended from the inoculation site to the bottom of the leaf in cultivar 02428, which was highly susceptible to seven *Xoo* races (Fig. 1e, Table S6). Therefore, it was suggested that the PXO99 might be highly virulent to *O. officinalis* as compared to C5.

Comparison of Leaf Microstructures Among Four *O. officinalis* Populations

The resistance of four *O. officinalis* populations to BB was different, while the numbers and area of vascular bundles in the leaf vein are related to the propagation and spreading velocity of *Xoo*. Therefore, we compared the leaf microstructures of four *O. officinalis* populations. We found that there were no significant differences in the microstructures of the leaf midvein and lateral vein among the four populations (Figs. 3 and 4). In addition, there was no remarkable difference in the number of macro-vascular bundles, small-vascular bundles, air cavities of leaf midvein, and leaf thickness among the four populations (Table 1, Fig. S1). The microstructures of the leaf midvein and lateral vein between the four populations and cultivar 02428 were significantly different (Figs. 3e and 4e). There were many bulging vascular bundles in the lateral vein of cultivar 02428 (Fig. 4e showed with elliptic ring), which were not found in *O. officinalis* populations. Although there were some differences in the arrangement of

Fig. 2 The comparison of leaf lesion area in four *O. officinalis* populations inoculated with seven *Xoo* races. The lesion area of the four populations was the largest after inoculation with PXO99, while the lesion area of the four populations was the smallest after inoculation with C5. The lesion area of the Gengma_Lincang population was the smallest among the four populations after inoculation with seven *Xoo* races. (**a**) Gengma, Lincang population; (**b**) Menghai, Xishuangbanna population; (**c**) Jingne, Jinghong population; (**d**) Lancang, Puer population; (**e**) cultivar 02428

vascular bundles between *O. officinalis* and cultivar 02428, there were no significant differences among the four populations. Interestingly, the area of macro-vascular bundles was not the smallest in the Gengma_Lincang population with the strongest BB resistance, while the area of macro-vascular bundles in the Jingne_Jinghong population was larger than that of the susceptible cultivar 02428 (Fig. S1b). These results suggested that the differences in BB resistance among the four *O. officinalis* populations might not be caused by the leaf microstructures but by the endogenous resistance genes.

Detection of Nine Cloned BB Resistance Genes in *O. officinalis* Populations

The PCR detection of *xa5*, *xa13*, *Xa21*, *Xa23*, and *Xa27* genes showed that *Xa21*, *Xa23*, and *Xa27* could not be detected any band, while *xa5* and *xa13* genes were amplified 170 and 280 bp susceptible bands, respectively, from the four *O. officinalis* populations (Fig. 5). The *Xa1*, *Xa10*, *Xa3/ Xa26*, and *xa25* genes were identified based on the primers designed by their ORF sequences. The results showed that only *Xa3/Xa26* was detected in the 1100 bp bands (Fig. 5), which were further confirmed to belong to the *xa3/xa26* homologous gene with 750 bp susceptible bands in the four populations (Fig. 6). Therefore, our results demonstrated that four *O. officinalis* populations possessed dominant *Xa5*, dominant *Xa13*, and recessive *xa3/xa26* homologous alleles of *xa5*, *xa13*, and *Xa3/Xa26* resistance genes.

The Cloning and BLAST Analysis of *OoXa5*, *OoXa13*, and *Ooxa3/xa26*

The *Xa5*, *Xa13*, and *xa3/xa26* homologous genes were isolated and cloned from the four populations (Fig. 7) and named *OoXa5*, *OoXa13*, and *Ooxa3/xa26*. The amino acid alignment showed that *OoXa5* belonged to *Xa5* susceptible gene for the 39th valine (V) (Fig. S2), which was consistent with the 170 bp susceptible band of PCR detection (Fig. 5). One amino acid was different at the 238th site of amino acid sequences between dominant *Xa13* and recessive *xa13*. Although *OoXa13* lacked the 238th and 237th sites of amino acids (Fig. S3), we detected 280 bp susceptible bands from the four populations using the primers designed by the different promoter regions between *Xa13* and *xa13* (Fig. 5). The 280 bp bands from the four populations were sequenced and aligned with the *Xa13* gene from IR64 (Fig. S4), which proved that the four populations carried the promoter regions of the *Xa13* gene. Therefore, we conjectured that *OoXa13* should be the allele of *xa13* rather than *xa13* resistance gene. However, the coding regions between *OoXa13* and reported *Xa13* were remarkably different, which indicated the BB resistance genes of *O. officinalis* might have its own characteristics, and
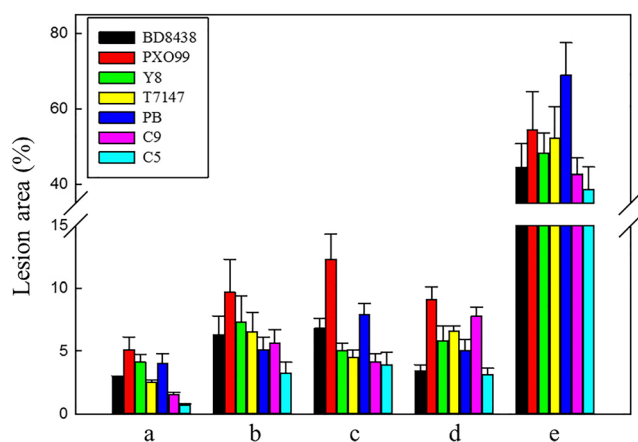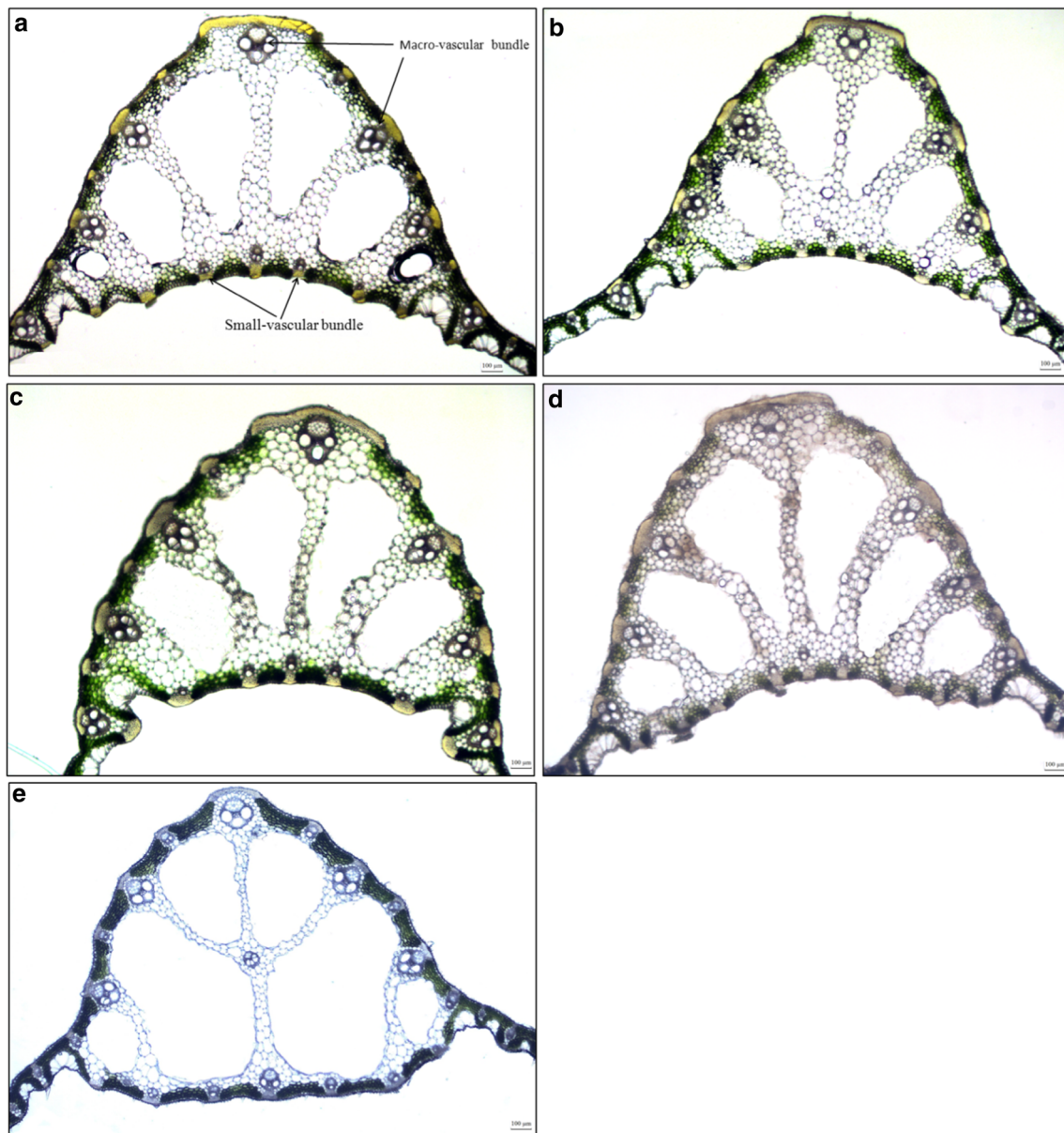
Fig. 3 The comparison of the microstructures in the leaf midvein among the four *O. officinalis* populations (50 ×). The middle part of the third leaf at the booting stage was chosen for observation of the leaf microstructures. There were no significant differences in the microstructures of the leaf midvein among the four populations. (**a**) Gengma, Lincang population; (**b**) Menghai, Xishuangbanna population; (**c**) Jingne, Jinghong population; (**d**) Lancang, Puer population; (**e**) cultivar 02428

O. officinalis might have new BB resistance genes and molecular mechanism different from the cultivated rice.

There are eight different amino acids between *xa3/xa26* and *Xa3/Xa26* in the LRR domains (Fig. S5, shown with *) (Hur et al. 2013). Except one amino acid, the other seven amino acids of *Ooxa3/xa26* were consistent with *xa3/xa26* in the LRR domains. The *Xa3/Xa26* resistance gene has the TGCA sequence in 452–456 bp from the start codon, whereas the recessive *xa3/xa26* has the AATC sequence at the same sites (Hur et al. 2013). *Ooxa3/xa26* had the AATC (429–432 bp) sequence as well as *xa3/xa26* at the corresponding sites (Fig. S6). According to the BLAST analysis of amino acids and nucleotides, *Ooxa3/xa26* from the four *O. officinalis* populations should belong to *xa3/xa26* homologous allele of *Xa3/Xa26* resistance gene, which was consistent with the 750 bp susceptible bands (Fig. 6).

Expression Profile Analysis of *OoXa5, OoXa13,* and *Ooxa3/xa26* in Response to *Xoo*

To evaluate the functions of *OoXa5, OoXa13,* and *Ooxa3/Ooxa26* in response to *Xoo*, we analyzed the expression patterns of them in *O. officinalis* under PXO99 and C5 stress. The expression levels of *OoXa13* and *Ooxa3/xa26* genes were
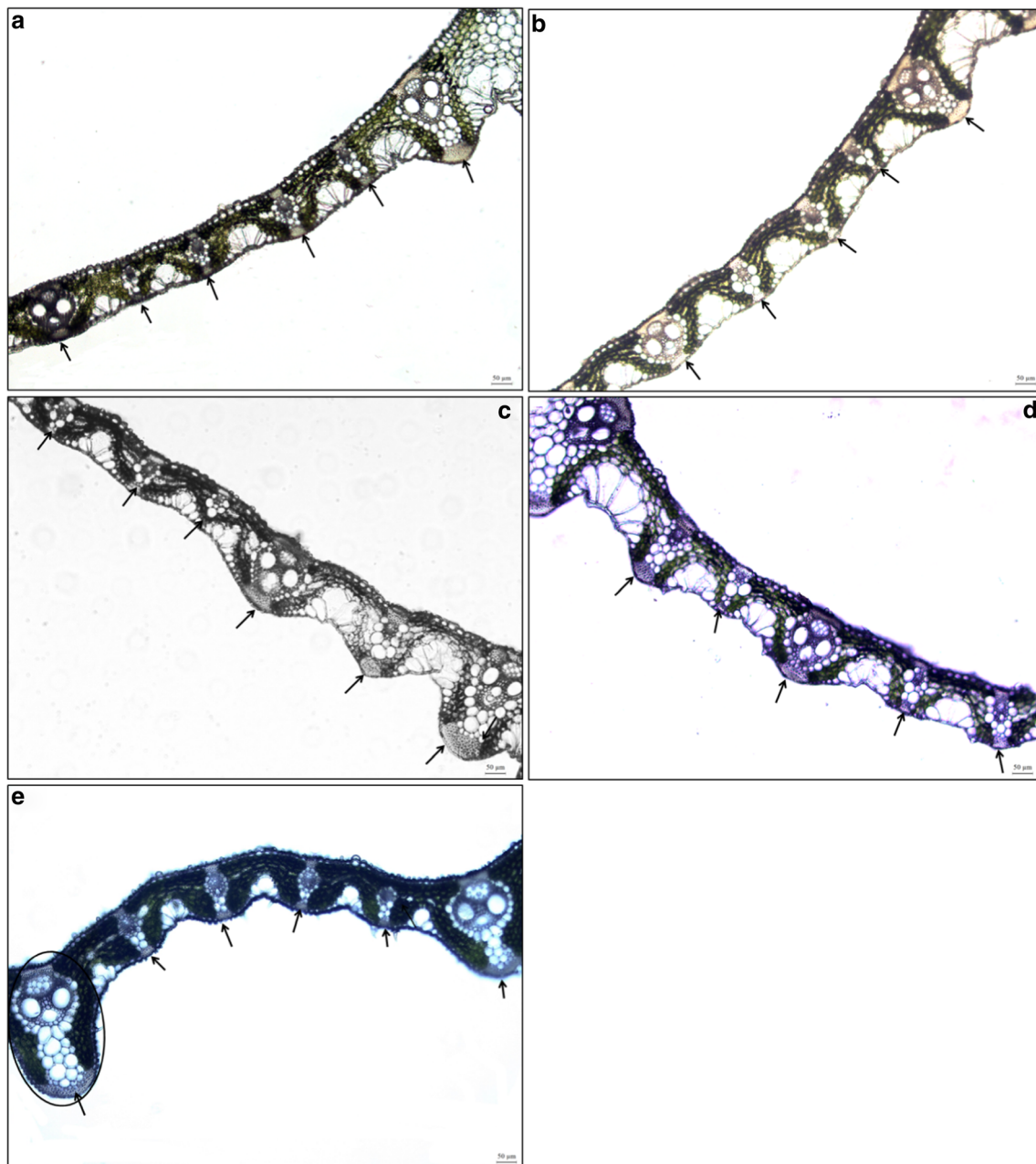
Fig. 4 The comparison of the leaf microstructures in the lateral vein among the four *O. officinalis* populations (100 ×). The middle part of the third leaf at the booting stage was chosen for observation of leaf microstructures. There were no remarkable differences in the microstructures of the lateral vein. (**a**) Gengma, Lincang population; (**b**) Menghai, Xishuangbanna population; (**c**) Jingne, Jinghong population; (**d**) Lancang, Puer population; (**e**) cultivar 02428

significantly down-regulated, while the expression of *OoXa5* was almost not significantly different under PXO99 and C5 stress (Figs. 8, 9, and 10). The whole expression levels of *OoXa5* were higher in CK that dealt with ddH₂O than those of under PXO99 and C5 stress (Fig. 8), which indicated that PXO99 and C5 might have no effect on the expression of *OoXa5* and the changes of *OoXa5* expression level might be caused by mechanical damage during the inoculation. The expression levels of *OoXa13* and *Ooxa3/xa26* were

significantly down-regulated under PXO99 and C5 stress, while their expression level was up-regulated in CK (Figs. 9 and 10). In addition, the expression levels of *OoXa13* and *Ooxa3/xa26* were lower in the Gengma_Lincang population than those of the three populations after inoculated with *Xoo*. Meanwhile, the *OoXa13* and *Ooxa3/xa26* expression levels continually decreased in the Gengma_Lincang population under C5 stress from 24 to 120 h (Figs. 9a and 10a). These results suggested that the expression levels of *OoXa13* and

Table 1 The comparison of the microstructures in the leaf midvein among the four *O. officinalis* populations

Populations	Number of MVB	Total area of MVB ($\times 10^2$ μm^2)	Number of SVB	Number of air cavity	Leaf thickness (μm)
Gengma, Lincang	5 ± 0	770.98 ± 30.71	9 ± 0.5	4 ± 0	131.03 ± 12.37
Menghai, Xishuangbanna	5 ± 0	680.54 ± 43.49	9.6 ± 0.8	4.8 ± 0.9	137.22 ± 15.72
Jingne, Jinghong	5 ± 0	1072.40 ± 22.90	11 ± 0	4 ± 0	138.73 ± 14.69
Lancang, Puer	7 ± 0	895.55 ± 43.02	10 ± 0	6 ± 0	145.04 ± 11.24
Cultivated rice 02428	6 ± 0	938.38 ± 28.23	11 ± 0.6	5.3 ± 1.0	154.39 ± 15.84

MVB, macro-vascular bundle; SVB, small-vascular bundle

Ooxa3/xa26 were remarkably suppressed in four *O. officinalis* populations under PXO99 and C5 stress, and the suppression effect was the most significant in the Gengma_Lincang population.

The Functional Domain Comparison Between *Ooxa3/xa26* and *Xa3/Xa26* or *xa3/xa26*

There were significant differences in the functional domains between *Ooxa3/xa26* from the four *O. officinalis* populations and *xa3/xa26* and *Xa3/Xa26* from cultivated rice (Fig. S7). The *xa3/xa26* (IR24) contained Pkinase and Pkinase_Tyr pfams at amino acid sequence of 800–1103 aa, while the *Xa3/Xa26* (Minghui63 and IRBB3) have three pfams of Pkinase, Pkinase_Tyr, and ABC1 at the same amino acid sequence sites. There was, however, only one type of S_TKc pfam in *Ooxa3/xa26* at the corresponding sites. In addition, there were three more LRR domains in *Ooxa3/xa26* than *xa3/xa26* and *Xa3/Xa26*. Interestingly, the domain of *Ooxa3/xa26* was LRR at the amino acid sites of 563–587 aa in Gengma_Lincang population, while it was LRRTYP in the other three populations. The difference of LRR in Gengma_Lincang was due to a different amino acid (Leucine, L) at 585 aa with CTT codon, while the amino acid was phenylalanine (F) with TTT codon in the other three populations.

Discussion

The BB Resistance of Gengma_Lincang Population Was the Strongest Among Four *O. officinalis* Populations

There are many reported BB resistance identification of *O. officinalis*. Yunnan Gengma *O. officinalis* has high resistance to BB (Li et al. 2015; Cheng et al. 2004; Peng et al. 1982). However, there is lack of systematical resistance identification of different Yunnan *O. officinalis* populations. In this study, seven representative hypervirulent *Xoo* races (Table S1) were chosen to identify the BB resistance of the four Yunnan *O. officinalis* populations from different original places, and some of the results were consistent with those of the previous studies. The four populations had strong resistance to seven *Xoo* races, but the resistance of the four populations was significantly different, in which the Gengma_Lincang population had the strongest resistance among them. In addition, the pathogenicity to the four populations of seven *Xoo* races was different, in which the pathogenicity of PXO99 was the strongest while C5 was the weakest. *Xoo* mainly invades the rice host through the leaf water stoma or wound and then propagates and spreads in the vascular bundle (Leach et al. 1989). Therefore, the numbers and area of vascular bundles of the
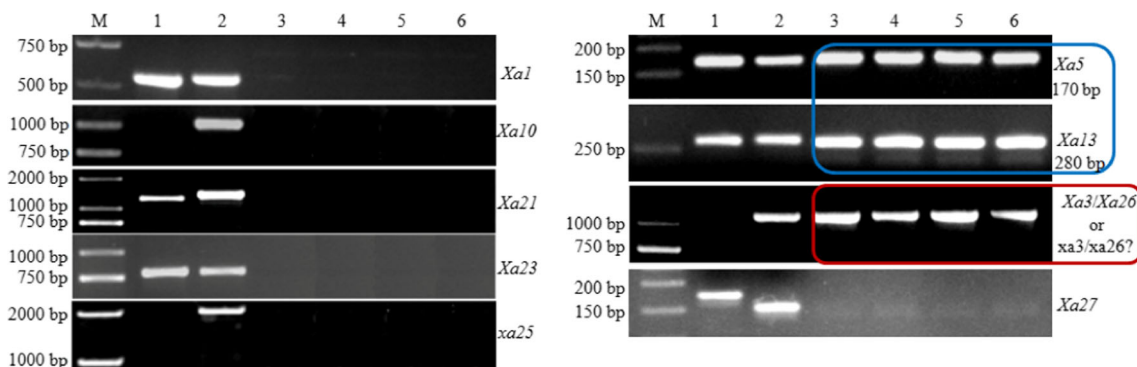

Fig. 5 The detection of nine cloned BB resistance genes in four *O. officinalis* populations. There were about 170 bp (*Xa5*) and 280 bp (*Xa13*) susceptible bands and 1100 bp *Xa3/Xa26* or *xa3/xa26* bands in the four populations. The other six BB resistance genes were not amplified any band from the four populations. (M) D2000 marker and 50 bp DNA ladder; (1) cultivar 02428; (2) positive controls with the corresponding BB resistance genes; (3) Gengma, Lincang; (4) Menghai, Xishuangbanna; (5) Jingne, Jinghong; (6) Lancang, Puer

Fig. 6 The detection of *Xa3/Xa26* and *xa3/xa26* in four *O. officinalis* populations. (**a**) There were about 750 bp bands belonging to *xa3/xa26* gene in the four populations. (**b**) There was no 255 bp resistance band of *Xa3/Xa26* in the four populations. (M) D2000 marker; (1) Gengma, Lincang; (2) Menghai, Xishuangbanna; (3) Jingne, Jinghong; (4) Lancang, Puer; (5) IR24 with *xa3/xa26*; (6) Minghui63 with *Xa3/Xa26*
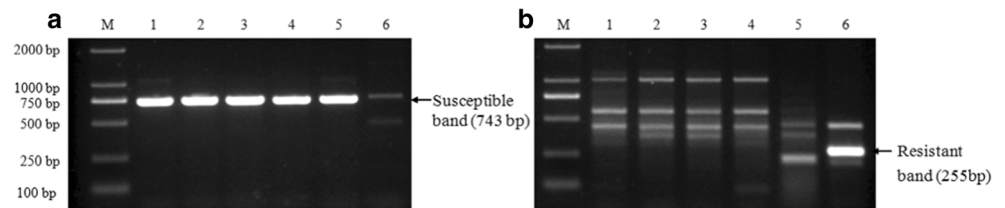
leaf might affect the propagation and spread velocity of *Xoo* in the leaf. We found that the resistance of the four populations to BB was significantly different, but there were no remarkable differences in their leaf microstructures, deducing the differences in BB resistance among the four populations were not caused by the leaf microstructures but by the endogenous resistance genes. The Gengma_Lincang population had the strongest resistance among the four populations, indicating this population might have a BB resistance gene family which conferred resistance specific to each BB race. Meanwhile, the expression levels of some key resistance genes might be induced under C5 stress and *O. officinalis* showed high resistance to C5. However, the resistance genes, no matter being induced by PXO99 or C5 in *O. officinalis*, might have strong resistance to other *Xoo* races.

There Might Be New BB Resistance Genes or Molecular Mechanism in *O. officinalis*

The different BB resistance among the four *O. officinalis* populations were determined by the internal resistance genes but not caused by their leaf microstructures. First of all, we detected nine cloned BB resistance genes to confirm whether they exist in the four *O. officinalis* populations. Li et al. (2015) found that *O. officinalis* possesses *Xa5* and *Xa13* homologous genes; however, it is not clear whether the *O. officinalis* contains *xa3/xa26* or *Xa3/Xa26*. In this study, we confirmed that the four *O. officinalis* populations possessed *Xa5*, *Xa13*, and *xa3/xa26* homologous genes, which were cloned from the four populations and named *OoXa5*, *OoXa13*, and *Ooxa3/xa26*, respectively. Our results proved that there were no nine cloned BB resistance genes in the four
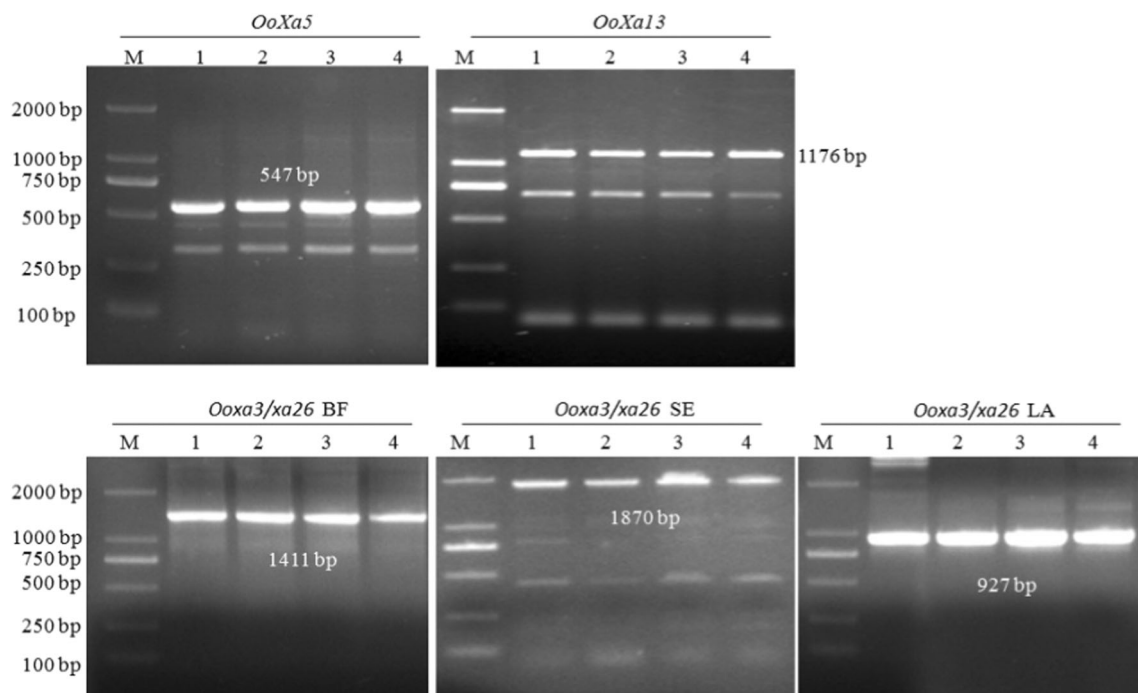

Fig. 7 The cloning of *OoXa5*, *OoXa13*, and *Ooxa3/xa26* from the four *O. officinalis* populations. (M) D2000 marker; (1) Gengma, Lincang; (2) Menghai, Xishuangbanna; (3) Jingne, Jinghong; (4) Lancang, Puer; (BF) the first fragment of *Ooxa3/xa26* ORF; (SE) the middle fragment of *Ooxa3/xa26* ORF; (LA) the last fragment of *Ooxa3/xa26* ORF
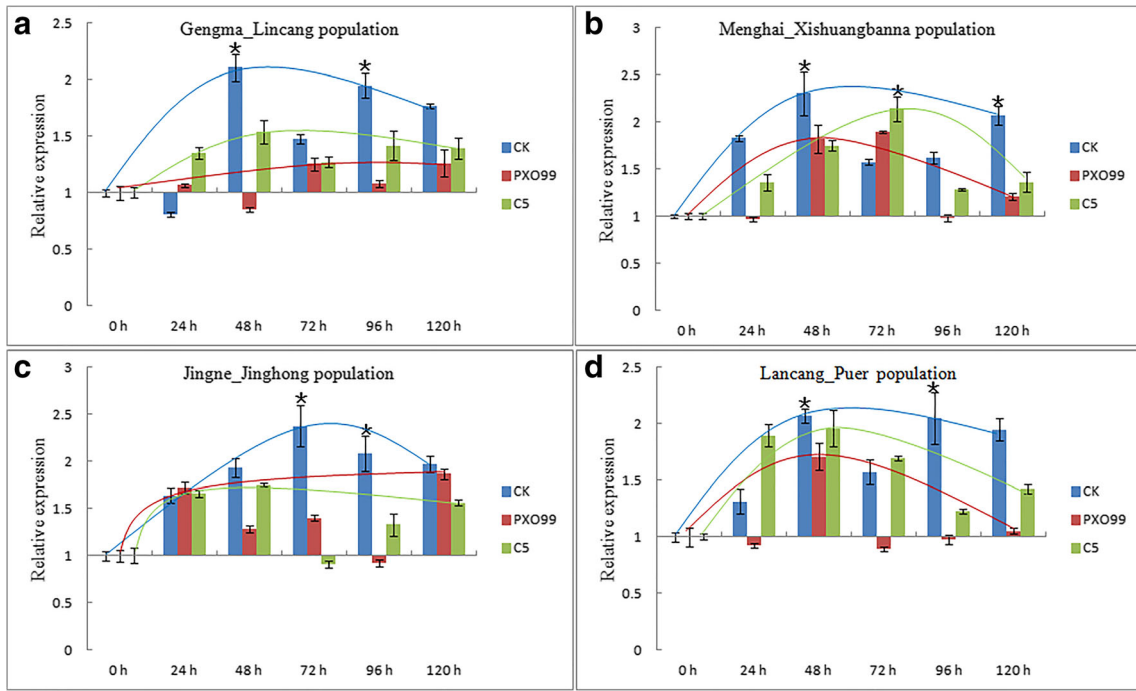
Fig. 8 The expression levels of *OoXa5* gene in the four *O. officinalis* populations under *Xoo* stress. The whole expression levels of *OoXa5* showed no significant change under PXO99 and C5 stress. * represented the expression levels of *OoXa5* at 24, 48, 72, 96, or 120 h

which were significantly different compared with 0 h ($p < 0.05$). The blue, red, and green curves represented the expression trend of *OoXa5* under ddH$_2$O, PXO99, and C5 stress, respectively. CK, ddH$_2$O treatment; PXO99, PXO99 stress treatment; C5, C5 stress treatment
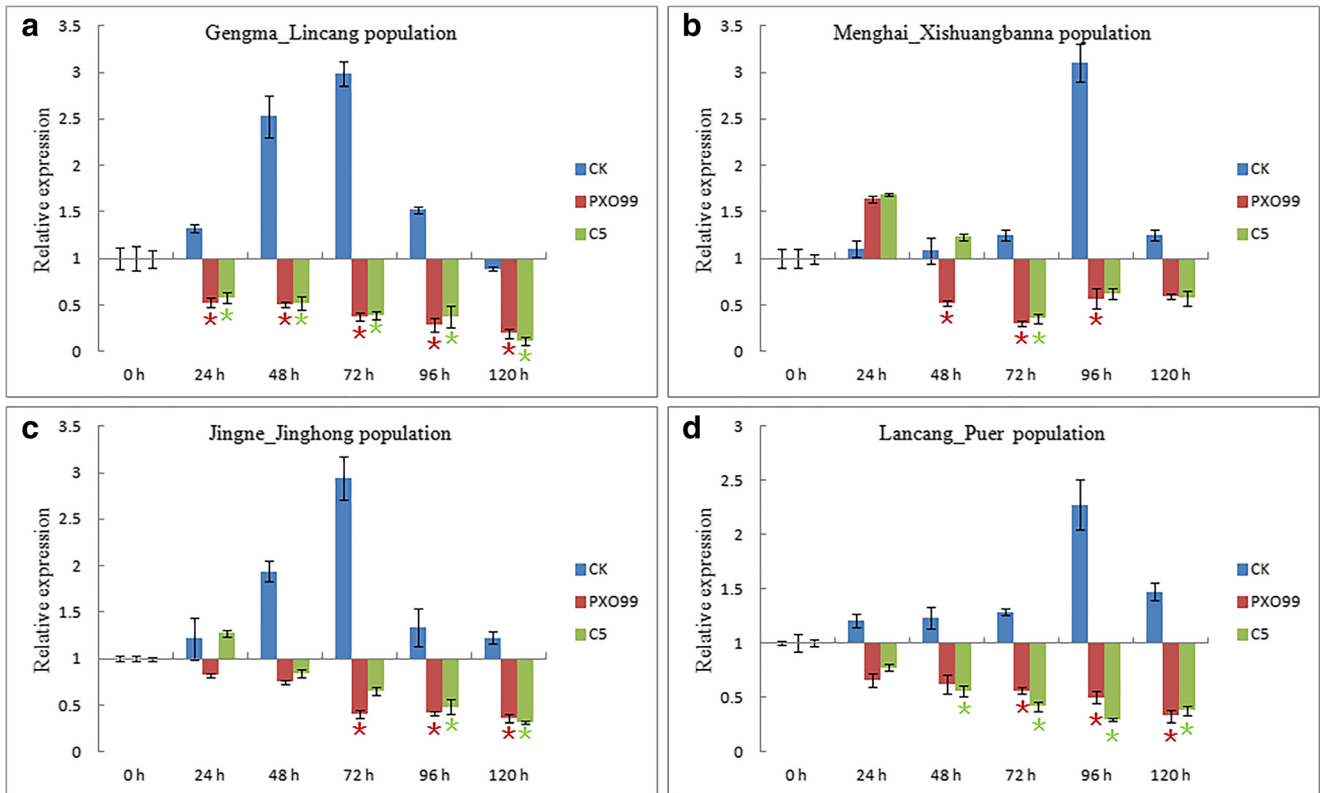

Fig. 9 The expression levels of *OoXa13* gene in the four *O. officinalis* populations under *Xoo* stress. The expression of *OoXa13* was significantly down-regulated under PXO99 and C5 stress. * represented

the expression levels of *OoXa13* at 24, 48, 72, 96, or 120 h which were significantly different compared with 0 h ($p < 0.05$). CK, ddH$_2$O treatment; PXO99, PXO99 stress treatment; C5, C5 stress treatment
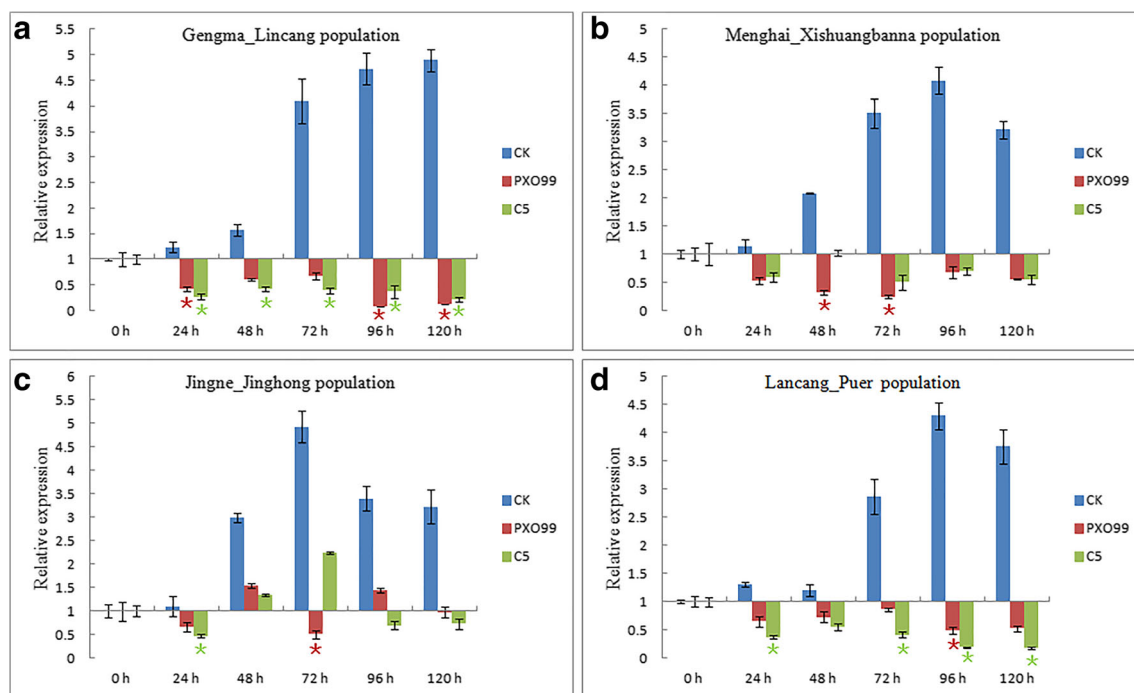
Fig. 10 The expression levels of *Ooxa3/xa26* gene in the four *O. officinalis* populations under *Xoo* stress. The expression of *Ooxa3/xa26* was significantly down-regulated under PXO99 and C5 stress. * represented the expression levels of *Ooxa3/xa26* at 24, 48, 72, 96, or 120 h which were significantly different compared with 0 h ($p < 0.05$). CK, ddH$_2$O treatment; PXO99, PXO99 stress treatment; C5, C5 stress treatment

populations. Thus, we concluded that the difference in BB resistance among the four populations were not caused by the leaf microstructures or the nine cloned BB resistance genes, which conjectured that *O. officinalis* might contain new BB resistance genes or molecular mechanism that was different from the cultivated rice.

The Contribution of *OoXa5*, *OoXa13*, and *Ooxa3/xa26* to the BB Resistance

Both *Xa5* and *xa5* genes have been shown previously to be constitutively expressed in rice leaf, and their expression levels were not remarkably different after inoculation with *Xoo* (Jiang et al. 2006; Iyer-Pascuzzi and McCouch 2004). The 39th glutamate of the dominant *Xa5* gene, exposed to the surface of the protein crystal structure, interacts with the avirulence protein, and, then, the Avrxa5 binds to the promoters of genes involved in susceptibility. Evidence has been presented that *Xa5* may be a nuclear target of *Xanthomonas* spp. which interacts with one or more *Xoo* TAL effectors, thus activating disease-promoting genes; however, the resistant protein xa5 in the *xa5* homozygote prevents the activation of the disease-promoting genes because there is no interaction between a *Xoo* effector and xa5, or such interaction is non-productive, and, then, the rice shows resistance to BB (Huang et al. 2016; Gu et al. 2009; Iyer-Pascuzzi et al. 2008; Sugio et al. 2007). The *xa5* is a completely recessive gene that mediates resistance in rice by restricting bacterial movement

(Iyer-Pascuzzi et al. 2008). Our study found that the whole expression levels of *OoXa5* were higher in CK that dealt with ddH$_2$O than those of under PXO99 and C5 stress and the PXO99 and C5 had no significant effect on the expression of *OoXa5*. This result suggested that the expression change of *OoXa5* under *Xoo* stress might be caused by mechanical damage during inoculation, which was consistent with that of the previous studies (Jiang et al. 2006; Iyer-Pascuzzi and McCouch 2004). However, whether the *OoXa5* would activate the expression of susceptible genes like *Xa5* gene and then affect the resistance to *Xoo* in *O. officinalis* needs further study.

The recessive *xa13* resistance gene is a novel *R* gene with resistance to *Xoo* race PXO99, but the expression of *xa13* was not affected by PXO99 (Yuan et al. 2010, 2011; Chu et al. 2006b). Suppression of *Xa13* expression significantly increases the resistance of rice to PXO99, and suppressing the expression of *xa13* further enhances the *xa13*-mediated resistance (Yuan et al. 2011; Chu et al. 2006b). Copper, an essential micronutrient of plants and an important element for a number of pesticides in agriculture, suppresses *Xoo* growth (Yuan et al. 2010). Xa13 protein cooperates with two other proteins, COPT1 and COPT5, to modulate copper redistribution and promote removal of copper from xylem vessels, where *Xoo* multiplies and spreads to cause disease (Yuan et al. 2010, 2011). PXO99 is more sensitive to copper than other *Xoo* races (Yuan et al. 2010, 2011). The expression levels of *OoXa13* in the four *O. officinalis* populations were suppressed significantly under

PXO99 and C5 stress. The suppressing expression of *OoXa13* would decrease the copper transport complexes of OoXa13 with COPT1 and COPT5, which would greatly reduce the removal of copper from the xylem vessels. Finally, the copper concentration in the xylem vessels was kept at high level which could effectively inhibit the propagation of *Xoo* and enhance the resistance of *O. officinalis* to PXO99 and C5. The expression level of *OoXa13* was the lowest and significantly suppressed in the Gengma_Lincang population. The lower expression level of *OoXa13* suggested that the copper transport complexes formed by OoXa13, COPT1, and COPT5 would be fewer in the Gengma_Lincang population and effectively suppress removal of copper from the xylem vessels. Therefore, we speculated that this might be one of the reasons that the BB resistance of the Gengma_Lincang population was stronger than that of the other three populations. The *xa13*-mediated resistant/susceptible signaling pathway does not belong to the types currently known and represents a new type of plant disease resistance (Chu et al. 2006b). The sequence alignment and analysis showed *OoXa13* from the four *O. officinalis* populations and *Xa13* of the cultivated rice had obvious differences (Fig. S3), suggesting *O. officinalis* might have its own resistant characteristic or new molecular mechanisms of BB resistance different from the cultivated rice.

Xa3/*Xa26* gene, like the most plant disease resistance genes, belongs to constitutive expression genes. Overexpression of *Xa3*/*Xa26* can enhance the resistance of transgenic plant to BB (Cao et al. 2007). The four *O. officinalis* populations contained recessive *xa3*/*xa26* homologous allele of *Xa3*/*Xa26* resistance gene. The expression levels of *Ooxa3*/*xa26* in the four populations were significantly down-regulated under PXO99 and C5 stress, especially in the Gengma_Lincang population. The inhibiting effect of PXO99 and C5 on *Ooxa3*/*xa26* expression was stronger in the Gengma_Lincang than the other three populations. However, there are no related reports that suppressing the expression of *xa3*/*xa26* can enhance the resistance of rice to BB. The functions of *Ooxa3*/*xa26*, which would enhance resistance to PXO99 and C5 by suppressing its own expression level like the *Xa13* gene, should be further studied.

The Effect on BB Resistance of *Ooxa3*/*xa26* LRR Domain in Gengma_Lincang *O. officinalis* Population

Sun et al. (2006) reported that the evolution of the *Xa3*/*Xa26* gene family is rapid and the point mutations of LRR involved in pathogen recognition are the major force of evolution. *Xa3*/*Xa26* belongs to a multigene family, consisting of *Xa3*/*Xa26*, *MRKa*, *MRKc*, and *MRKd*. Complementary analysis showed that *MRKa* and *MRKc* cannot mediate resistance to BB when regulated by their native promoters, but *MRKa* confers partial resistance to BB when regulated by a strong constitutive promoter; *MRKd* however is a pseudogene (Xu et al. 2008; Cao

et al. 2007). Overexpression of *MRKa* can enhance partial resistance of rice to BB (Cao et al. 2007). Although *MRKa* and *MRKc* cannot mediate BB resistance, they may be once effective genes for *Xoo* resistance. Some members of the *Xa3*/*Xa26* gene family once may lose the resistance due to the evolution and variation of the *Xoo*, and then these genes regain resistance and become new resistance genes for point mutations (Xu et al. 2008; Cao et al. 2007). These results indicated that the *Xa3*/*Xa26* gene family is constantly evolving to respond to the variation of pathogen, suggesting there may be some resistance genes of the *Xa3*/*Xa26* gene family that have not yet been identified or cloned.

LRR participates in the identification of different physiological races to determine the resistant specificity of the *Xa3*/*Xa26* gene (Zhao et al. 2009). The domain was LRR at the sites of 563–587aa in the Gengma_Lincang *Ooxa3*/*xa26*, whereas it was LRRTYP in the other populations and cultivar at the corresponding sites. The difference in LRR of the Gengma_Lincang *Ooxa3*/*xa26* was caused by one different amino acid (leucine, L) at 585 aa with CTT codon, while the amino acid was phenylalanine (F) with TTT codon in the other materials. One-point mutation of *Ooxa3*/*xa26* caused a domain change, which might change the BB resistance level of *Ooxa3*/*xa26*. Based on the previous studies (Zhao et al. 2009; Cao et al. 2007), the change of the LRR domain in the Gengma_Lincang *Ooxa3*/*xa26* might be due to the point mutations when the LRR identified the pathogen. The expression level of *Ooxa3*/*xa26* in the Gengma_Lincang was the lowest under PXO99 and C5 stress among the four populations. Whether this was related with the changed LRR domain or would the *Ooxa3*/*xa26* of the Gengma_Lincang regain the resistance and become a new BB resistance gene during co-evolution with the pathogen should be further studied.

Conclusion

The BB resistance genes of *O. officinalis* had its own characteristic, and there might be new BB resistance genes and molecular mechanism in *O. officinalis*. First, there were no remarkable differences in the leaf microstructures among the four *O. officinalis* populations, which indicated that the difference in BB resistance of the four populations was caused by the resistance genes. Second, the four populations exhibited various BB resistance, among which the Gengma_Lingcang population had the strongest resistance. Four populations possessed dominant *Xa5*, dominant *Xa13*, and recessive *xa3*/*xa26* homologous alleles of *xa5*, *xa13*, and *Xa3*/*Xa26* resistance genes. Third, the expression levels of *Xa5*, *Xa13*, and *xa3*/*xa26* were different in the four populations under *Xoo* stress. *OoXa13* could enhance the resistance to PXO99 and C5 by down-regulating its own expression level, while the contribution of *Ooxa3*/*xa26* on BB resistance might function like *OoXa13*.

This study provided a solid foundation and reference for studying the molecular mechanism of BB resistance in *O. officinalis*.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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