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Exploring the mechanism and efficient use of a durable gene-mediated resistance to bacterial blight disease in rice

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Abstract *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) causes bacterial blight, the most devastating bacterial disease of rice worldwide. The major disease resistance gene *Xa3/Xa26* confers a durable resistance to *Xoo* with a dosage effect. However, the mechanism of *Xa3/Xa26*-mediated resistance remains to be elucidated. We created near-isogenic lines carrying *Xa3/Xa26* with a background of *indica* and *japonica*, the two major subspecies of Asian cultivated rice. Analyzing these rice lines showed that the *japonica* background facilitated resistance to *Xoo*, which was associated with increased *Xa3/Xa26* expression, compared with rice lines with an *indica* background. This characteristic of *Xa3/Xa26* was related to the *WRKY45* locus, which had higher expression with the *japonica* background than with the *indica* background. However, the two alleles of the *WRKY45* locus had different expression levels, with the *WRKY45-1* expression level being higher than that of *WRKY45-2* for both *japonica* and *indica* backgrounds. In addition, the resistance level conferred by *Xa3/Xa26* was higher in the presence of *WRKY45-1* than in the presence of *WRKY45-2* for both *japonica* and *indica* backgrounds. *Xa3/Xa26*-mediated resistance was associated with increased accumulation of jasmonic acid (JA), JA-isoleucine, and terpenoid and flavonoid phytoalexins. Exogenous JA application enhanced *Xa3/Xa26*-mediated resistance. These results not only provide more knowledge toward understanding the mechanism of *Xa3/Xa26*-mediated resistance but also offer the best choice for using *Xa3/Xa26* for rice resistance improvement, specifically, a *japonica* background with the *WRKY45-1* allele.

Keywords Disease · Durable resistance · *indica* · *japonica* · *Oryza sativa* · *Xanthomonas oryzae*

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Introduction

Plant disease resistance can be classified as being either genetic or molecular. At the level of genetics, it can be divided into qualitative (complete) resistance conferred by major disease resistance (*MR*) genes and quantitative

(incomplete) resistance mediated by quantitative trait loci (QTLs) or multiple defense-related genes (Kou and Wang 2010; Zhang and Wang 2013; Ke et al. 2017). At the molecular level, disease resistance can be explained by a two-tiered innate immune system consisting of pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl 2006). In general, the PTI initiated by plasma membrane-localized pattern recognition receptors (PRRs) is a weak or quantitative resistance, and ETI initiated by cytoplasm-localized nucleotide-binding–leucine-rich repeat (NB-LRR) disease resistance (R) proteins is a race-specific high level of resistance or qualitative resistance (Jones and Dangl 2006; Dodds and Rathjen 2010). However, strong PTI and weak ETI also exist (Thomma et al. 2011; Zhang and Wang 2013).

Bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is the most devastating bacterial disease in rice worldwide. One of the most efficient and cost-effective ways to control *Xoo* infection is to develop rice with broad-spectrum and durable disease resistance (Kou and Wang 2010). Rice qualitative resistance to *Xoo* can be conferred by *MR* gene *Xa21*. *Xa21* encodes plasma membrane-localized LRR receptor kinase, which belongs to the largest class of PRRs, and confers a broad-spectrum and strong PTI to *Xoo* (Song et al. 1995; Monaghan and Zipfel 2012; Pruitt et al. 2015). Another rice *MR* gene *Xa3/Xa26* conferring *Xoo* resistance encodes a similar protein (Sun et al. 2004; Xiang et al. 2006), suggesting that it may also confer PTI to *Xoo*. Furthermore, *Xa3/Xa26* has a *Xoo* resistance spectrum that differs from *Xa21*, and it confers a broad-spectrum and durable disease resistance at both seedling and adult stages (Zhao et al. 2009; Gao et al. 2010; Li et al. 2012). This gene has played an important role in rice production in China for a long time (Gao et al. 2010), but the mechanism of *Xa3/Xa26*-mediated resistance to *Xoo* remains to be elucidated. Exploring the mechanism of *Xa3/Xa26*-mediated resistance may provide breeders a better way to use this gene.

Previous study has revealed that *Xa3/Xa26* has a dosage effect: the higher its expression, the more resistant the rice plant (Cao et al. 2007). Asian cultivated rice consists of two major groups (subspecies), *indica* and *japonica*. *Xa3/Xa26*, which was first characterized in *indica* cultivar Minghui 63, functions better with a *japonica* background than with an *indica* background in resistance to *Xoo*. This resistance is associated with a higher level of *Xa3/Xa26* expression throughout the growth stages based on the study of transgenic plants with a *japonica* background carrying transgene *Xa3/Xa26* driven by its native promoter (Sun et al. 2004; Cao et al. 2007). However, it is not clear whether this higher level of *Xa3/Xa26* expression represents its natural expression level, for *Cauliflower mosaic virus 35S* promoter in the transformation construct aiming to regulate marker gene may have a residual effect on the expression of the target transgene (Yoo et al. 2005; Zheng et al. 2007; Singer et al. 2010; Yang et al. 2011).

Phytohormones play important roles in plant–pathogen interactions (Fu and Dong 2013). Salicylic acid (SA) and/or jasmonic acid (JA) have been reported to be associated with some types of defense-responsive gene-regulated resistance to *Xoo* (Qiu et al. 2007; Yuan et al. 2007; Tao et al. 2009; Xiao et al. 2009; Shen et al. 2010, 2011; Deng et al. 2012; Ke et al. 2014; Hu et al. 2017). However, indole-3-acetic acid (IAA), the main type of auxin in plants, and abscisic acid (ABA) promote rice susceptibility to *Xoo* (Ding et al. 2008; Fu et al. 2011; Xu et al. 2013). Phytoalexins, which are low-molecular-weight compounds with antimicrobial activity, are a group of plant disease resistance products produced after pathogen infection (Van Etten et al. 1994). Previous study revealed that *Xa3/Xa26*-mediated resistance to *Xoo* is associated with increases in SA and JA levels and the flavonoid phytoalexin content but not the common terpenoid phytoalexin content in comparison to a susceptible cultivar with an *indica* rice background (Liu et al. 2012). However, it is unknown whether defense-related phytohormones and phytoalexins contribute to genetic background-influenced function of *Xa3/Xa26* against *Xoo*.

To address these uncertainties, we generated both *japonica* and *indica* near-isogenic lines carrying *Xa3/Xa26* by continuous backcrossing and used resistant and susceptible rice lines with the same genetic background to compare the levels of resistance and the concentrations of phytohormones and metabolites putatively involved in plant–pathogen interactions. Our results suggest that the *japonica* background does facilitate the resistance function of *Xa3/Xa26*, which is associated with increased expression of *Xa3/Xa26*, compared with the *indica* background. *Xa3/Xa26*-mediated resistance to *Xoo* with a *japonica* background is more strongly associated with JA than SA and a dramatic increase of both terpenoid and flavonoid phytoalexins, but there is no obvious association with reinforcement of the cell

wall. In addition, genetic background-influenced function of *Xa3/Xa26* is associated with the *WRKY45* locus, and this locus can also slightly reduce rice susceptibility to *Xoo* strain that is compatible to *Xa3/Xa26*.

Materials and methods

Rice materials

We generated two sets of rice near-isogenic lines containing *MR* gene *Xa3/Xa26* plus allelic gene *WRKY45-1* or *WRRY45-2* by using *indica* rice cultivar (*Oryza sativa* ssp. *indica*) Minghui 63 and *japonica* rice cultivar (*O. sativa* ssp. *japonica*) Mudanjiang 8 as donor and also recurrent parents. Minghui 63, moderate resistance to *Xoo*, was used as a donor parent of *Xa3/Xa26* and *WRKY45-2* genes, and Mudanjiang 8, susceptible to *Xoo*, was used as a donor parent of *WRKY45-1*. MD1 and MD2 were *japonica* Mudanjiang 8 near-isogenic lines containing *Xa3/Xa26*, which was created by backcrossing (BC) eight times, using Minghui 63 as a donor parent and Mudanjiang 8 as a recurrent parent. To start, Mudanjiang 8 was crossed with Minghui 63 to produce an F_2 population. In this population, plants that appeared similar to Mudanjiang 8 and carried *Xa3/Xa26* and *WRKY45-1* or *WRKY45*-2 were chosen as male parents to backcross with Mudanjiang 8 to produce BC_1F_1 plants. The BC_1 plants were consecutively backcrossed with Mudanjiang 8 seven times to generate BC_8F_1 plants. MD1 and MD2 were BC_8F_3 lines; MD1-1 and MD1-2 carried *Xa3/Xa26* and *WRKY45-1*, and MD2-1 and MD2-2 carried *Xa3/Xa26* and *WRKY45-2*. MH1 and MH2 were *indica* Minghui 63 near-isogenic lines, which were created by the same backcross method, using Mudanjiang 8 as a donor parent and Minghui 63 as a recurrent patent. MH1 and MH2 were BC_6F_3 lines; MH1-1 and MH1-2 carried *Xa3/Xa26* and *WRKY45-1*, and MH2-1 and MH2-2 carried *Xa3/Xa26* and *WRKY45-2*. Three pairs of primers were used to examine the existence of *Xa3/Xa26*, *WRKY45-1*, or *WRKY45-2* in near-isogenic lines (Supplemental Table 1).

Transgenic line Rb49, which has high resistance to *Xoo*, was generated by transforming *Xa3/Xa26* driven by its native promoter into *japonica* rice cultivar Mudanjiang 8 (Sun et al. 2004; Xiang et al. 2006). The *indica* cultivar Minghui 63 carrying *Xa3/Xa26* has moderate resistance to *Xoo* (Sun et al. 2004; Liu et al. 2011). The Wase Aikoku 3 is a *japonica* rice cultivar in which *Xa3/Xa26* has been first identified (Ezuka et al. 1975). The IRBB3 is an *indica* near-isogenic rice line carrying *Xa3/Xa26* gene (Ogawa et al. 1988; Xiang et al. 2006).

Disease evaluation

The leaves of rice plants were inoculated with *Xoo* strains PXO61 and PXO99 by the leaf-clipping method at the seedling (six-leaf) or the booting (panicle development) stage (Chen et al. 2002). Disease was scored by measuring the lesion length (cm) at 14 days after inoculation.

Hormone treatment

Rice plants were grown in the greenhouse until the six-leaf stage. Benzothiadiazole (BTH) (250 μM BTH [B10900, Sigma] in 0.05% [*v*/*v*] methanol plus 0.05% [*v*/*v*] Tween 20), JA (250 μM JA [J2500, Sigma] in 0.05% (*v*/*v*) methanol plus 0.05% [*v*/*v*] Tween 20), or BTH plus JA (250 μM JA and 250 μM BTH in 0.05% [*v*/*v*] methanol plus 0.05% [*v*/*v*] Tween 20) was foliar sprayed until runoff. A solution containing 0.05% (*v*/*v*) methanol and 0.05% (*v*/*v*) Tween 20 was used as the mock-treatment control. The sprayed plants remained sealed in separated transparent plastic shades for 24 h before and after treatment, respectively. The plants were then inoculated with *Xoo* strain PXO61.

Quantification of phytohormone and metabolite

Approximately 3-cm-long fragments next to the bacterial inoculation sites were collected from flag leaves at the booting stage. Samples were prepared and quantified using the ultrafast liquid chromatography–electrospray ionization tandem mass spectrometry system as previously reported (Liu et al. 2012).

Gene expression analysis

Quantitative reverse transcription (qRT)-PCR analysis was conducted as described previously (Qiu et al. 2007). PCR primers are listed in Supplementary Table 1. The expression level of the rice actin gene was used as an internal control. The expression level relative to control is presented. Each analysis was repeated biologically at least twice with similar results, and each biological

repeat had three technical replicates. Only one repeat is presented.

Statistical analysis

Statistical analysis between two samples was performed using Student's *t* test in Excel (Microsoft, Redmond, WA). Statistical analysis among multiple samples was performed by one-way ANOVA using Tukey's multiple comparison test in software R (The R project for Statistical Computing; https://www.r-project.org). The correlation analysis was also performed using R software.

Results

Analysis of the effect of *japonica* background on *Xa3/Xa26*-mediated resistance to *Xoo*

To find out if a higher expression level of *Xa3/Xa26* is necessary for improved resistance with a *japonica* background, we transferred *Xa3/Xa26* from the *indica* cultivar Minghui 63 into the susceptible *japonica* cultivar Mudanjiang 8 by continuous backcrossing, using Mudanjiang 8 as a recurrent parent. The resulting near-isogenic line MD1 had similar plant height, morphology, and growth period as the Mudanjiang 8 (Fig. 1a). The transgenic rice line Rb49, which carries a single copy of transgene *Xa3/Xa26* driven by its native promoter with the genetic background of Mudanjiang 8 (Sun et al. 2004; Cao et al. 2007), also has morphology and a growth period similar to Mudanjiang 8 (Fig. 1a). Consistent with previous results (Sun et al. 2004; Xiang et al. 2006; Cao et al. 2007), Minghui 63 had moderate resistance to *Xoo* strain PXO61 with an average lesion length 16-fold longer than that of Rb49 (Fig. 1b). MD1 had high resistance to PXO61, but its average lesion length was approximately threefold longer than that of Rb49. Meanwhile, Rb49 showed the highest expression level of *Xa3/Xa26* among the three rice lines, and Minghui 63 had the lowest (Fig. 1b). The *Xa3/Xa26* expression level in MD1 was also significantly higher ($P < 0.01$) than that in Minghui 63 but obviously lower than that of Rb49 (Fig. 1b). These results suggest that a *japonica* background facilitates the resistance function of *Xa3/Xa26*, which is associated with significantly increased ($P < 0.01$) expression of *Xa3/Xa26*. However, in comparison with MD1, the expression level of *Xa3/Xa26* in transgenic line Rb49 does not represent its natural expression level and the *35S* promoter in the transformation construct may also induce *Xa3/Xa26* expression.

Previous studies report that multiple QTLs affect the genetic background-controlled disease resistance conferred by *Xa3/Xa26* (Zhou et al. 2009). The peak region of a major QTL, which explained 17% of the phenotypic variation of resistance to *Xoo* strain PXO61, co-localizes with the defense-responsive *WRKY45* locus; this resistance QTL is contributed by the allele from susceptible Mudanjiang 8 (Zhou et al. 2009; Kou et al. 2010). The *WRKY45* locus has two alleles, *WRKY45-1* and *WRKY45-2*, which encode WRKY-type transcription factors that differ by 10 amino acids, and *WRKY45-1* but not *WRKY45*-2 can generate small interfering RNA (siRNA) from its intron (Tao et al. 2009; Zhang et al. 2016). Three *japonica* rice varieties (including susceptible Mudanjiang 8) carry the *WRKY45-1* allele and three *indica* rice varieties (including moderately resistant Minghui 63) carry the *WRKY45-2* allele; both *WRKY45-1* and *WRKY45-2* function in the *Xa3/Xa26*-initated defense signaling pathway (Tao et al. 2009; Zhou et al. 2009; Kou et al. 2010). The MD1 line carries *WRKY45-1* in addition to *Xa3/Xa26* (Fig. 1a). To examine whether the *WRKY45* locus was associated with genetic background-influenced *Xa3/Xa26* function during *Xoo* infection, we also generated near-isogenic line MD2, which carried *WRKY45-2* in addition to *Xa3/Xa26* with the genetic background of Mudanjiang 8 (Fig. 1a). Another set of near-isogenic lines MH1 and MH2 carried *WRKY45-1* and *WRKY45-2*, respectively, with the genetic background of Minghui 63 carrying *Xa3/Xa26* (Fig. 1a). The rice lines MD1 and MD2 had significantly more resistance to *Xoo* than the rice lines MH1, MH2, and Minghui 63 (Fig. 2a). Compared with susceptible Mudanjiang 8, both MD1 and MD2 lines were resistant to *Xoo* (Fig. 2a), but MD1 plants were significantly more resistant ($P < 0.01$) than MD2 plants. A similar result was also observed in near-isogenic lines with Minghui 63 background. Both Minghui 63 and MH2 plants showed a similar level of moderate resistance to *Xoo*, but MH1 plants had a significantly higher level ($P < 0.01$) of resistance to *Xoo* than MH2 plants.

We have proved that *japonica* Mudanjiang 8 benefits *Xa3/Xa26*-mediated resistance against *Xoo* compared with *indica* Minghui 63-mediated resistance against *Xoo* (Figs. 1b and 2a). To ensure this situation also exists in other cultivars, we checked a *japonica* Wase Aikoku 3

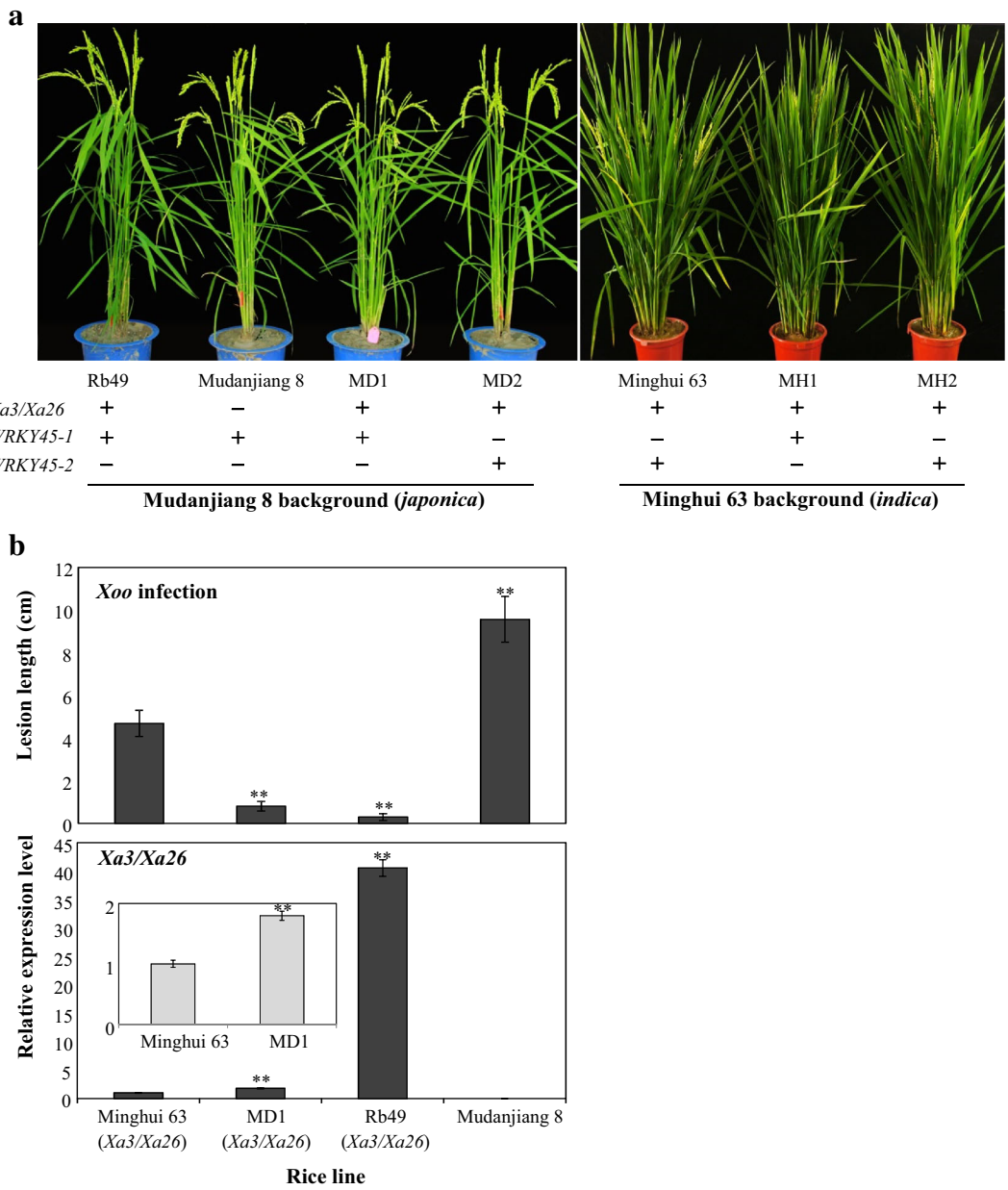

Fig. 1 The phenotype of rice lines carrying *Xa3/Xa26* with a *japonica* and an *indica* backgrounds. **a** The morphology of different rice lines selectively carrying the major disease resistance gene *Xa3/Xa26* and the defense-responsive allelic gene *WRKY45-1* or *WRKY45-2*. Susceptible Mudanjiang 8 is the recurrent parent of near-isogenic lines MD1 and MD2. Rb49 is a transgenic line carrying transgene *Xa3/Xa26* driven by its native promoter. Moderately resistant Minghui 63 is the recurrent parent of near-isogenic lines MH1 and MH2. **b** The response of different rice lines to *Xoo* strain PXO61 infection and the expression of *Xa3/Xa26*. Plants were inoculated with *Xoo* at the booting stage and disease was scored at 14 days after inoculation. The RNA samples were got before *Xoo* infection at the booting stage. Bars represent mean (8 to 13 leaves from three to five plants for lesion length and three replicates for gene expression) ± standard deviation (SD). The asterisks above bars indicate a significant difference between Minghui 63 and other rice lines at $P < 0.01$

cultivar and an *indica* IRBB3 line. Sequence analysis of genetic identified *Xa3/Xa26* in Wase Aikoku 3 (Ezuka et al. 1975) showed this gene (including 3.4 kb genome region and 2 kb promoter region) having identical sequence (GenBank accession numbers MG641894 and MG641895 for Wase Aikoku 3) to the *Xa3/Xa26* gene

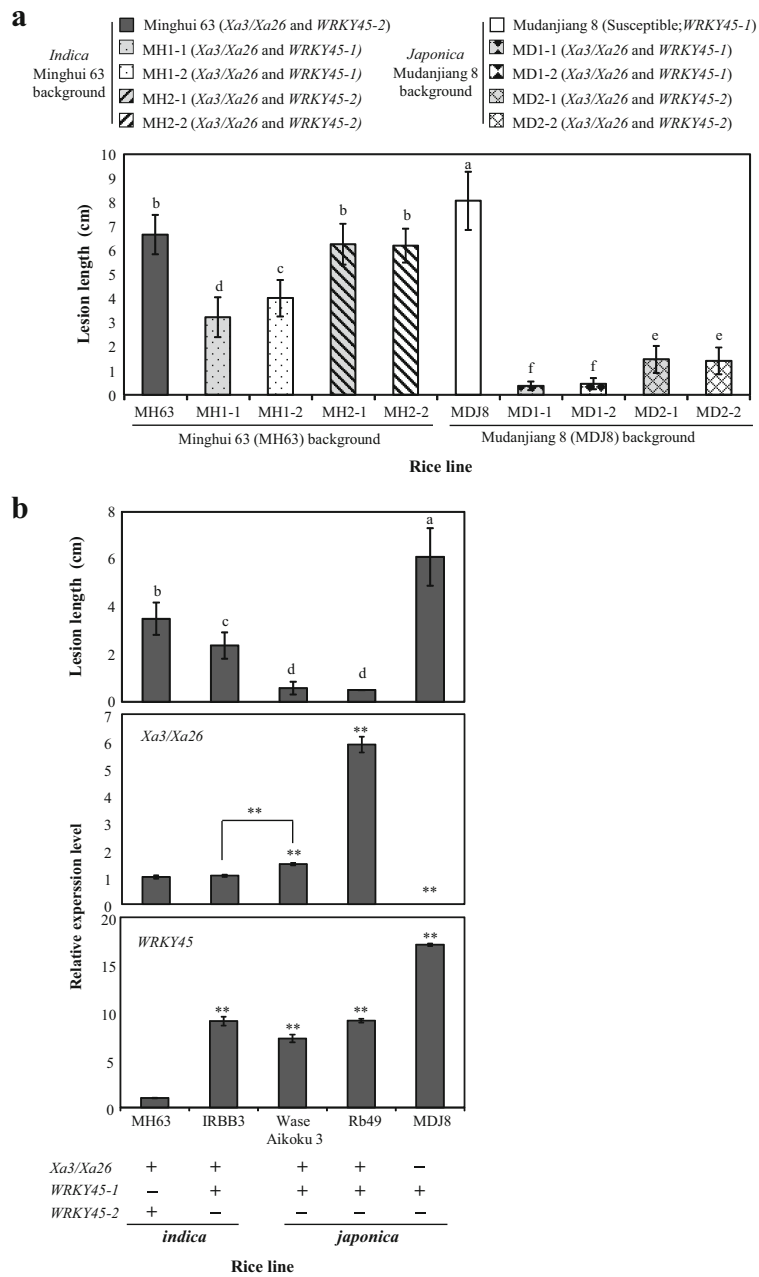

Fig. 2 The resistance levels of different rice lines to *Xoo*. Plants were inoculated with *Xoo* strain PXO61 for 14 days at the booting stage. Bars represent mean (15 to 20 plants for **a** and at least 10 leaves from two or three plants for **b**) ± SD. Different letters above the bars indicate significant differences at $P < 0.01$. **a** The resistance levels of different Mudanjiang 8 and Minghui 63 near-isogenic lines. **b** The resistance levels of three different *japonica* and two *indica* lines. The asterisks above the bars indicate a significant difference between Minghui 63 and other cultivars or between two cultivars linked by lines at $^{**}P < 0.01$

in IRBB3 and Minghui 63 (Sun et al. 2004; Xiang et al. 2006). The Wase Aikoku 3-mediated resistance to *Xoo* was similar to *japonica* Rb49, but higher than IRBB3 and Minghui 63 (Fig. 2b). Furthermore, *Xa3/Xa26* expression level in Wase Aikoku 3 was significantly ($P < 0.01$) higher than that in IRBB3 and Minghui 63 (Fig. 2b). Interestingly, we found *indica* line IRBB3 carried *WRKY45-1* (Fig. 2b). Consistent with this result, IRBB3-mediated resistance to *Xoo* was significantly ($P < 0.01$) higher than Minghui 63-mediated resistance (Fig. 2b). These results reproved that *japonica* Wase Aikoku 3 also can facilitate the resistance function of *Xa3/Xa26* to *Xoo* compared with *indica* rice line IRBB3 and Minghui 63. Furthermore, *WRKY45* alleles may also influence *Xa3/Xa26* function in other rice lines, such as *indica* IRBB3 and Minghui 63.

In addition to having higher levels of *Xa3/Xa26* expression, MD1 and MD2 had higher levels of

WRKY45 expression (either *WRKY45-1* or *WRKY45-2*) than Minghui 63, MH1, and MH2 (Fig. 3). However, the expression levels of *Xa3/Xa26* between MD1 and MD2 and between MH1 and MH2 showed no obvious differences, but the expression levels of *WRKY45-1* in MD1 and MH1 were significantly higher ($P < 0.01$) than those of *WRKY45-2* in MD2 and MH2 both before and after *Xoo* infection (Fig. 3). Consistent with these results, the expression levels of *Xa3/Xa26* between *indica* IRBB3 and Minghui 63 showed no obvious differences; however, expression level of *WRKY45-1* in IRBB3 was significantly higher ($P < 0.01$) than that of *WRKY45-2* in Minghui 63 (Fig. 2b). As transcription activators (Shimono et al. 2007; Cheng et al. 2015), neither WRKY45-1 nor WRKY45-2 interacted with the *Xa3/Xa26* promoter in yeast cells, although this promoter harbors seven W and W-like boxes, putatively for the binding of WRKY transcription factors. Rice *ST1* gene, encoding a LRR receptor kinase-like protein, is an important component in *WRKY45*-mediated base defense in susceptible rice (Zhang et al. 2016). In order to know whether the *ST1* expression was associated with different genetic background, we checked its expression in rice lines carrying *Xa3/Xa26*. MD1 and MD2 had higher levels of *ST1* expression than MH1 and MH2 in general (Fig. 3). However, the expression levels of *ST1* between MD1 and MD2 and between MH1 and MH2 showed no obvious differences. In addition, in the rice lines carrying *Xa3/Xa26*, the expression of *ST1* was significantly correlated ($r = 0.512$, $n = 36$, $P < 0.01$) with the expression of *WRKY45* (Fig. 3).

In order to know whether *WRKY45* locus only influences the resistance function of *Xa3/Xa26* or not, we checked the near-isogenic lines with another *Xoo* strain PXO99, which is compatible with *Xa3/Xa26* (Sun et al. 2004; Cao et al. 2007). In both Minghui 63 and Mudanjiang 8 backgrounds, near-isogenic lines carrying *WRKY45-1* were significantly less susceptible to PXO99 ($P < 0.01$) than lines carrying *WRKY45-2*, although these near-isogenic lines were still susceptible to PXO99 (Supplemental Fig. 1).

All these results suggest that the *japonica* background also facilitated the expression of *WRKY45* and *ST1*, which is associated with increased *Xa3/Xa26*-mediated resistance to *Xoo*. However, expression of *Xa3/Xa26* facilitated by a *japonica* background may not be directly related to *WRKY45* activation. *Xa3/Xa26* in combination with *WRKY45-1* can mediate a higher level of resistance than *Xa3/Xa26* in combination with *WRKY45-2* with either an *indica* or a *japonica* background. The result also suggests that *WRKY45-1* can slightly promote rice resistance to *Xoo* strain (such as PXO99) that is compatible to *Xa3/Xa26*. Thus, using *Xa3/Xa26* in combination with *WRKY45-1* in a *japonica* background is the best way to improve rice resistance to *Xoo*.

Analysis of phytohormone response in *Xa3/Xa26*-mediated resistance to *Xoo* in *japonica* background

To explore the mechanism of how the *japonica* background facilitates *Xa3/Xa26*-mediated resistance to *Xoo*, more analyses were carried out as follows. SA and JA in combination or alone have been reported to be associated with rice resistance to *Xoo* mediated by different defense-related genes (Qiu et al. 2007; Shen et al. 2010; Hu et al. 2017). To clarify which hormone defense signaling contributes to *Xa3/Xa26*-mediated resistance with a *japonica* background, we quantified the levels of hormones commonly involved in plant–pathogen interactions in resistance rice lines MD1 and Rb49, which carry *WRKY45-1* but differing levels of *Xa3/Xa26* transcripts, and susceptible line Mudanjiang 8. Consistent with previous reports (Ding et al. 2008; Fu et al. 2011; Liu et al. 2012; Ke et al. 2014), rice basal SA level was much higher than the levels of JA, ABA, and IAA (Fig. 4a). No obvious difference of the SA level before and after *Xoo* infection was detected in any of the rice lines. However, the SA levels in resistance lines were significantly higher than in the susceptible line at 10 and 72 h after *Xoo* infection. JA and JA-isoleucine (Ile) are collectively known as jasmonates. The jasmonate levels in the three rice lines showed a similar pattern in response to *Xoo* infection, except that the JA level was approximately threefold higher than the JA-Ile level (Fig. 4a). The jasmonate levels were significantly induced ($P < 0.01$) in both resistant and susceptible lines. However, their levels were higher in resistant lines than in the susceptible line during early infection (2 h), lower in resistant lines than in the susceptible line at 10 and 24 h after infection, and higher again in resistant lines than in the susceptible line at 48 and 72 h. Furthermore, the jasmonate levels were much higher in Rb49 than in MD1 at 72 h after infection.

ABA and IAA increase rice susceptibility to *Xoo* (Ding et al. 2008; Fu et al. 2011; Xu et al. 2013). To examine whether *Xa3/Xa26*-mediated resistance was

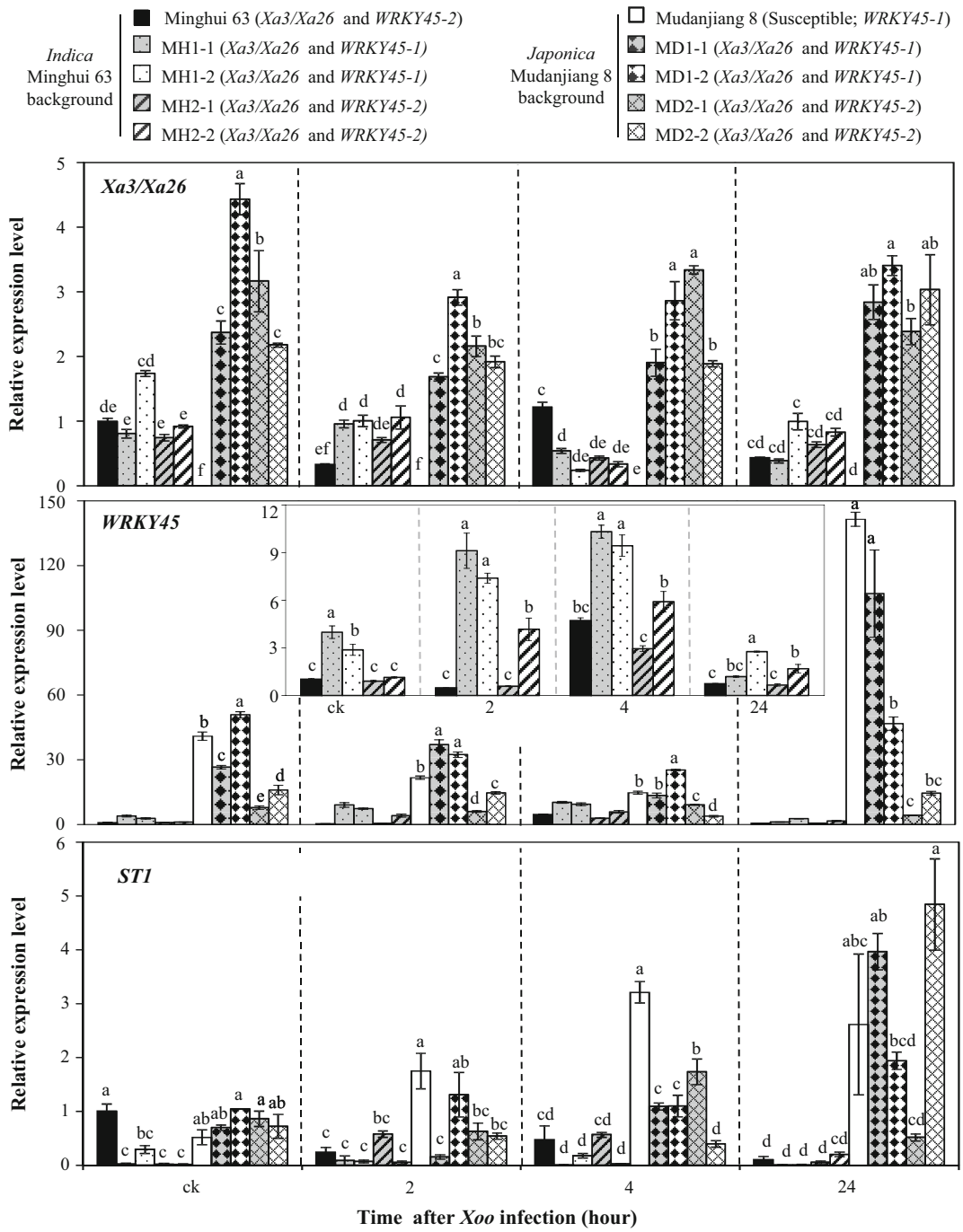

Fig. 3 The expression of *Xa3/Xa26*, *WRKY45*, and *ST1* in different rice lines with Minghui 63 or Mudanjiang 8 background before (ck) and after *Xoo* strain PXO61 infection. Different letters above bars indicate a significant difference at the same time point at $P < 0.01$ analyzed by following two methods. (1) The significant difference of *Xa3/Xa26* and *ST1* expression at the same time point was analyzed using 10 rice lines including five *indica* lines and five *japonica* lines. (2) The significant difference of *WRKY45* expression in five *indica* lines and five *japonica* lines at the same time point was separately analyzed, because the high expression level of *WRKY45* in *japonica* lines will cover the expression difference in five *indica* lines

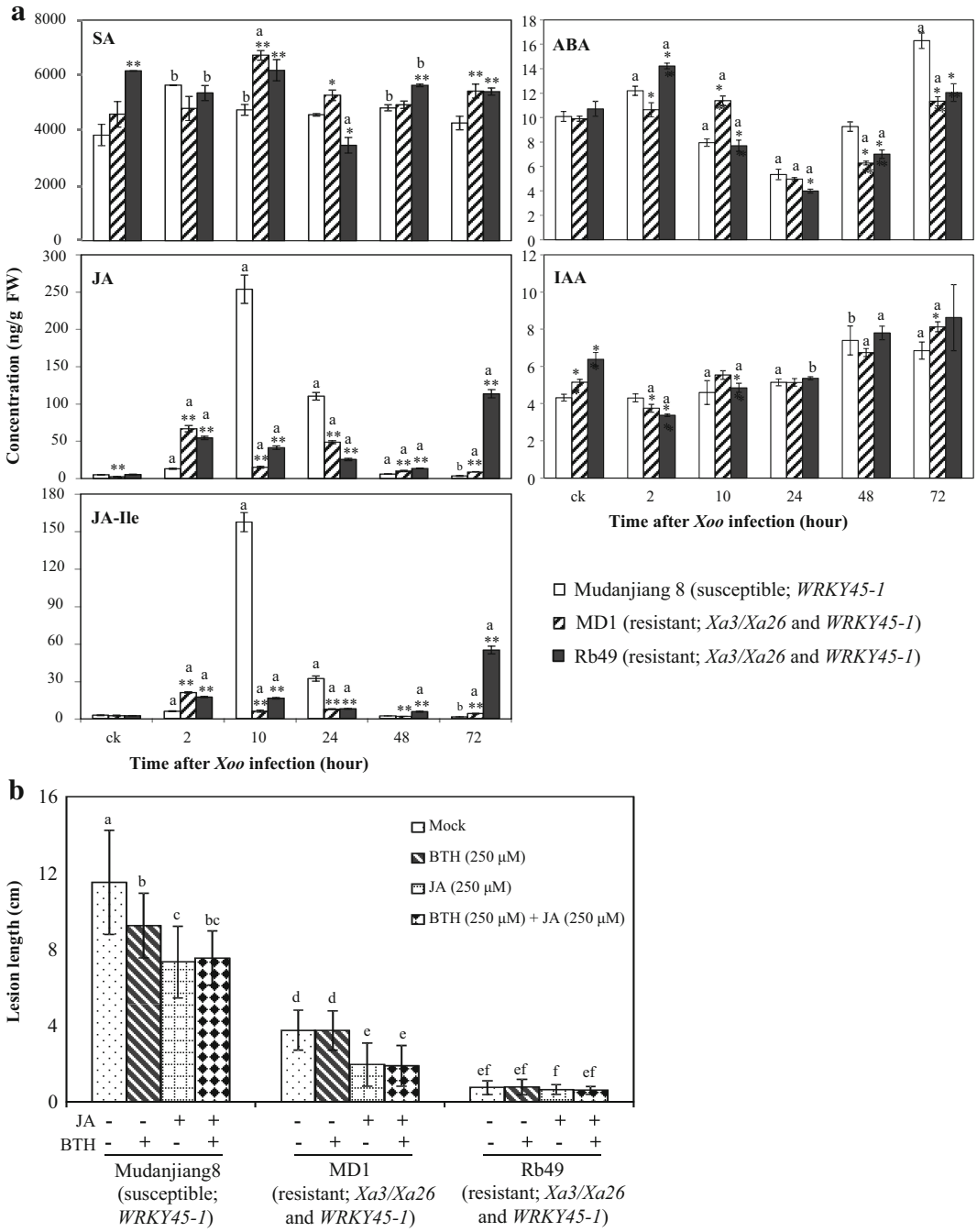

Fig. 4 The interactions between phytohormones and *Xa3/Xa26*-mediated resistance. Plants were inoculated with *Xoo* strain PXO61. **a** The concentrations of endogenous phytohormones before and after *Xoo* infection in different rice lines with a Mudanjiang 8 background. The asterisks above the bars indicate a significant difference between wild type and MDl or Rb49 at the same time point at $^{**}P < 0.01$ or $^{*}P < 0.05$. The letter a or b above the bar indicates a significant difference between before (ck) and after *Xoo* infection in the same rice line at $P < 0.01$ or $P < 0.05$. **b** Effects of BTH and JA on rice response to *Xoo* infection. Plants were inoculated with *Xoo* after 24 h of treatment with BTH, JA, or BTH plus JA. Bars represent mean (12 to 51 plants) ± SD. Different letters above bars indicate significant differences at $P < 0.01$

associated with suppressed accumulation of ABA and IAA, we qualified the levels of the two hormones in the same samples used for qualifying SA and jasmonates. In response to *Xoo* infection, both ABA and IAA levels showed a similar pattern in the three rice lines (Fig. 4a). However, the ABA level was significant lower ($P < 0.01$) in the two resistant lines than in the susceptible line at 48 and 72 h after *Xoo* infection. The IAA levels between the resistant and susceptible rice lines showed no obvious difference after *Xoo* infection (Fig. 4a). In summary, these results suggest that SA and jasmonates may be involved in rice resistance at different time points after *Xoo* infection of plants with the *japonica* background, and this resistance may be also associated with suppressed accumulation of ABA.

Analysis of the effects of SA and JA on *Xa3/Xa26*-mediated resistance

As a functional analog of SA, BTH can activate systemic acquired resistance in both monocots and dicots (Görlach et al. 1996; Lawton et al. 1996; Lee et al. 2013). As an efficient inducer of resistance, BTH has been widely used in rice instead of SA (Fitzgerald et al. 2004; Shimono et al. 2007, 2012; Bai et al. 2011; Ueno et al. 2015). To examine whether SA and JA play roles in *Xa3/Xa26*-initiated resistance, we analyzed the effects of BTH and exogenous JA application on rice resistance to *Xoo*. Consistent with previous findings (Shimono et al. 2012; Ke et al. 2014), BTH and JA pretreatments significantly reduced ($P < 0.01$) the susceptibility to *Xoo* in susceptible Mudanjiang 8, compared to a mock control (Fig. 4b). However, JA treatment showed a stronger effect than BTH treatment on Mudanjiang 8, and JA-treated Mudanjiang 8 had a significantly shorter lesion length ($P < 0.01$). Furthermore, pretreatment with both BTH and JA did not have additive effect on Mudanjiang 8 to *Xoo* infection. The BTH had no effect on resistant lines MD1 and Rb49, and BTH-treated plants had similar lesion lengths as mock-treated plants after *Xoo* infection (Fig. 4b). JA had a significant effect ($P < 0.01$) on MD1, and the average lesion length of the JA-treated MD1 plants was approximately half of that of mock-treated plants. However, JA showed no effect on Rb49 (Fig. 4b). This finding may be due to Rb49 having high resistance to *Xoo* and already containing a high level of endogenous jasmonates compared with MD1 (Fig. 4a). These results suggest that jasmonate involved defense signaling may play an important role in *Xa3/Xa26*-mediated resistance.

Analysis of the factors directly against pathogen invasion

Upon pathogen infection, a plant displays various defense responses. The cell wall is reinforced to slow down pathogen invasion or avoid second invasion, and the plant produces toxic chemicals such as phytoalexins to kill the invading pathogens (Agrios 2005). To study which final direct factors are involved in *Xa3/Xa26*-mediated resistance against *Xoo* invasion, we analyzed the concentration of phytoalexins in rice plants responding to *Xoo*. The terpenoid phytoalexin momilactone A, the flavonoid phytoalexin sakuranetin, and the precursor of sakuranetin, naringenin, are common phytoalexins associated with rice resistance to *Xoo*, although they are not always present (Padmavati et al. 1997; Qiu et al. 2008; Liu et al. 2012; Ke et al. 2014; Duan et al. 2016; Hu et al. 2017). The momilactone A levels in the susceptible Mudanjiang 8 and resistant MD1 and Rb49 were much higher than sakruranetin and naringenin levels both before and after *Xoo* infection (Fig. 5). *Xoo* infection significantly induced the accumulation of molitactone A, sakruranctin, and naringenin in all the three lines. However, the levels of the three phytoalexins were 3-, 31-, and 44-fold higher in MD1 than in Mudanjiang 8, and 3-, 74-, and 65-fold higher in Rb49 than in Mudanjiang 8 at 5 days after *Xoo* infection (Fig. 5).

Expansins are proteins that loosen the cell wall (Cosgrove 2005). Rice *EXPA1*, *EXPA5*, and *EXPA10* genes encode α-expansins, and activating these genes increases rice susceptibility to *Xoo*. Resistance to *Xoo* mediated by rice genes *GH3-8*, *GH3-2*, and *Xa4* is associated with suppressed expression of *EXPA1*, *EXPA5*, and *EXPA10* (Ding et al. 2008; Fu et al. 2011; Hu et al. 2017). Cellulose is a major element of the cell wall, and it is synthesized by enzymes of the cellulose synthase (CESA) family (Somerville 2006). Rice *CesA4*, *CesA7*, and *CesA9* genes are responsible for the secondary cell wall synthesis (Zhang and Zhou 2011). Rice *MR* gene *Xa4*-mediated resistance to *Xoo* is associated with increased expression of *CesA4*, *CesA7*, and *CesA9* (Hu et al. 2017). To study whether cell wall reinforcement contributes to *Xa3/Xa26*-mediated resistance, we analyzed the expression of the above-mentioned genes in response to *Xoo* infection.

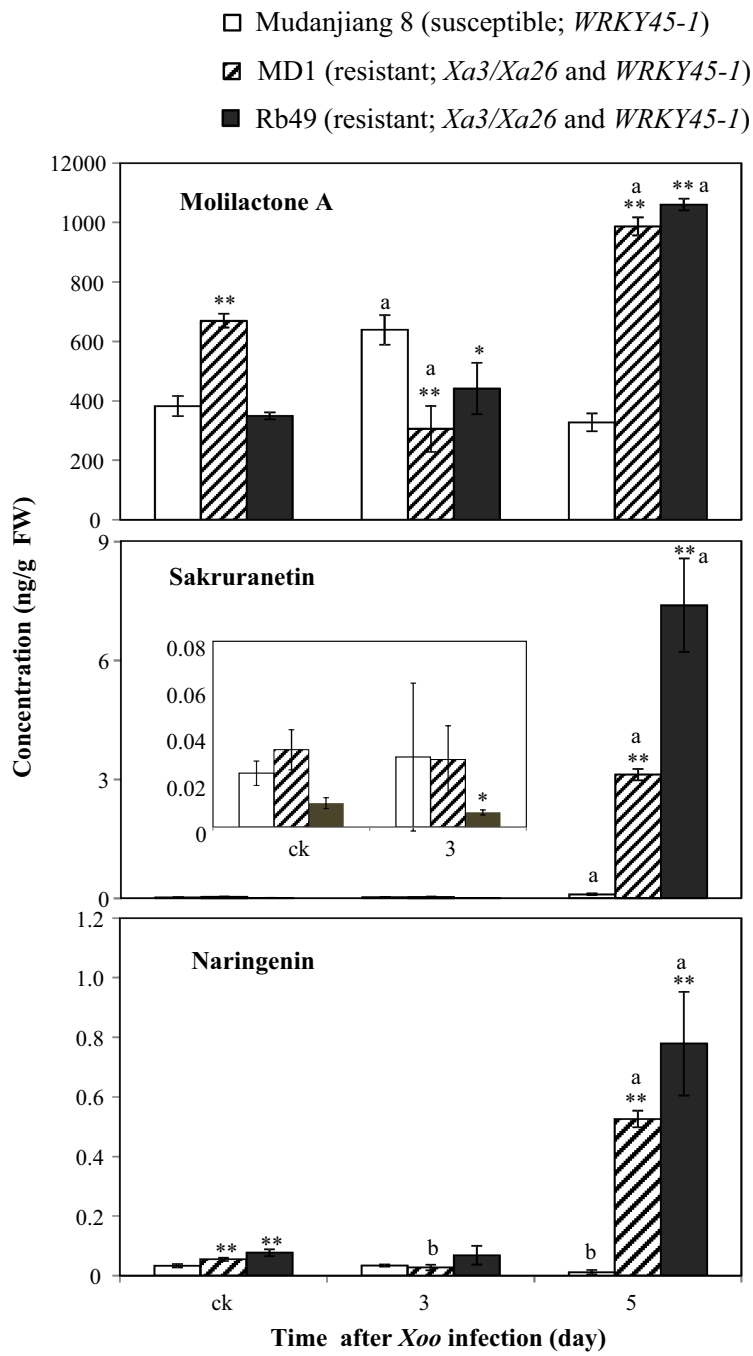

Fig. 5 The concentrations of phytoalexins before and after *Xoo* strain PXO61 infection in different rice lines with a Mudanjiang 8 background. The asterisks above the bars indicate a significant difference between Mudanjiang 8 and MDl or Rb49 at the same time point at $^{**}P < 0.01$ or $^{*}P < 0.05$. The letter a or b above the bar indicates a significant difference between before (ck) and after *Xoo* infection in the same rice line at $P < 0.01$ or $P < 0.05$

In general, the expression of *EXPA1*, *EXPA5*, and *EXPA10* in resistant MD1 and Rb49 lines showed similar or even higher levels than in susceptible Mudanjiang 8 (Supplemental Fig. 2). Only the expression of *CesA4* and *CesA9* was significantly induced in both resistant lines compared to susceptible Mudanjiang 8 at 2 h after *Xoo* infection among the time points examined. In addition, *CesA4*, *CesA7*, and *CesA9* showed significantly higher expression levels in Rb49 at 10 h after infection than in MD1 and Mudanjiang 8 (Supplemental Fig. 2). At the other time points examined, *CesA4*, *CesA7*, and *CesA9* showed similar or even lower expression levels in resistant lines than in the susceptible line (Supplemental Fig. 2). These results suggest that an

accumulation of phytoalexins, but not an obvious strengthening of cell wall, contributes to *Xa3/Xa26*-mediated resistance to *Xoo* in plants with a *japonica* background.

Discussion

Although several genes functioning in the *Xa3/Xa26*-initiated defense signaling pathway have been identified, which reveals some characteristics of *Xa3/Xa26*-mediated disease resistance (Qiu et al. 2007; Tao et al. 2009; Xiao et al. 2009; Deng et al. 2012; Xiao et al. 2013), the mechanism of *Xa3/Xa26*-mediated resistance to *Xoo* remains to be elucidated. Based on previous studies, the current work offers insight in several ways.

First, we validated that a *japonica* background can enhance *Xa3/Xa26*-mediated resistance to *Xoo* by promoting *Xa3/Xa26* expression compared with an *indica* background. In a previous study, we examined the response of *japonica* transgenic lines carrying *Xa3/Xa26* driven by its native promoter to *Xoo* infection and found that *Xa3/Xa26* has a dosage effect; the rice *japonica* background facilitated *Xa3/Xa26*-mediated resistance, which was associated with markedly increased expression of *Xa3/Xa26*, compared with the *Xa3/Xa26* donor of the *indica* cultivar Minghui 63 (Cao et al. 2007). However, it is uncertain whether the expression level of *Xa3/Xa26* in the transgenic lines represents a natural expression level. In this study, we compared near-isogenic lines carrying *Xa3/Xa26* in both *japonica* cultivar Mudanjiang 8 and *indica* Minghui 63 backgrounds and showed that Mudanjiang 8 background facilitates *Xa3/Xa26* expression and promotes rice resistance to *Xoo*. This result is consistent with a previous report that *Xa3/Xa26* is an important resistance gene in *japonica* cultivars planted in China (Xu et al. 2004). In addition, the present results suggest that the *WRKY45* locus appears to be associated with *Xa3/Xa26* expression facilitated by Mudanjiang 8 background. The *WRKY45* locus functions in the *Xa3/Xa26*-initiated defense pathway (Tao et al. 2009; Zhou et al. 2009), and both WRKY45-1 (named WRKY45 by Shimono et al. (2007)) and WRKY45-2 are transcription activators (Shimono et al. 2007; Cheng et al. 2015). The two *WRKY45* alleles showed higher expression levels in plants with Mudanjiang 8 background than in those with Minghui 63 background as the expression of *Xa3/Xa26* (Fig. 3). However, the near-isogenic lines carrying *Xa3/Xa26* and *WRKY45-1* had a higher resistance level than those carrying *Xa3/Xa26* and *WRKY45-2* with both Mudanjiang 8 and Minghui 63 backgrounds. The resistance was associated with the *WRKY45-1* expression level being higher than the *WRKY45-2* expression level but not obviously associated with the *Xa3/Xa26* expression level (Fig. 3). These results suggest that WRKY45 may not directly regulate *Xa3/Xa26* expression. This hypothesis is also supported by the finding that neither WRKY45-1 nor WRKY45-2 interacted with *Xa3/Xa26* promoter in yeast cells.

Although the present results are mainly from analyzing two sets of near-isogenic lines, one *japonica* Mudanjiang 8 background and one *indica* Minghui 63 background, previous reports support that other *japonica* and *indica* cultivars' backgrounds also influence *Xa3/Xa26* function. *Xa3/Xa26* driven by its native promoter was also transformed into another two *japonica* cultivars (carrying WRKY45-1), Zhonghua 11 and 02428 (Cao et al. 2007). These *japonica Xa3/Xa26*-transgenic lines with Zhonghua 11 or 02428 background had higher level of resistance to *Xoo* than that of *indica* Minghui 63, and this high level of resistance was associated with increased expression of *Xa3/Xa26*. Furthermore, *indica* rice line IRBB3 also carries *Xa3/Xa26* (Xiang et al. 2006). These *Xa3/Xa26*-transgenic lines with Zhonghua 11 or 02428 background showed higher resistance to *Xoo* than that of IRBB3 (Cao et al. 2007). We also examined a *japonica* Wase Aikoku 3 cultivar and an *indica* IRBB3 line both carrying *Xa3/Xa26*. The Wase Aikoku 3-mediated resistance to *Xoo* was significantly higher ($P < 0.01$) than IRBB3 and also Minghui 63 (Fig. 2b). Furthermore, *Xa3/Xa26* expression level in Wase Aikoku 3 was significantly higher ($P < 0.01$) than that in IRBB3 and Minghui 63 (Fig. 2b). All the results suggest that different *japonica* cultivars may suit higher efficient use of *Xa3/Xa26* against *Xoo*.

In susceptible rice that does not carry *Xa3/Xa26*, activation of the *WRKY45-1* gene leads to the rice plants being more susceptible to *Xoo*. This activation is associated with suppression of the *ST1* gene, while activation of *WRKY45-2* gene reduces rice susceptibility to *Xoo*, which is associated with increase of *ST1* expression (Tao et al. 2009; Zhang et al. 2016). However, in the same susceptible background, activation of the modified *WRKY45-1* gene, in which siRNA-generating insertion in the intron is omitted, results in reduced susceptibility to *Xoo* associated with increase of *ST1* (Zhang et al. 2016). This result is due to the siRNA

being able to suppress *ST1* by RNA-directed DNA methylation. However, in the rice lines carrying *Xa3/Xa26*, the expression of *ST1* was significantly correlated ($P < 0.01$) with expression of *WRKY45* (Fig. 3). Thus, further study is required to determine whether *WRKY45-1* may function differently in susceptible and resistant rice backgrounds.

Second, jasmonate signaling plays an important role in *Xa3/Xa26*-mediated resistance. Among 40 named *MR* genes against *Xoo*, only *Xa3/Xa26* and *Xa4* have been reported to confer durable resistance (Leach et al. 2001; Li et al. 2012; Hu et al. 2017; Ke et al. 2017). *Xa4* encodes a cell wall-associated kinase, and reinforcing the cell wall contributes to *Xa4*-mediated resistance (Hu et al. 2017). This resistance is associated with increased accumulation of JA-Ile but not of JA. *Xa3/Xa26*-mediated resistance is likely through PTI as *MR* gene *Xa21*-mediated resistance to *Xoo* (Zhao et al. 2009; Ke et al. 2017). In contrast, *Xa3/Xa26*-mediated resistance is associated with increased accumulation of both JA and JA-Ile. Thus, *MR* gene-mediated durable resistance to *Xoo* can be regulated by different signaling pathways.

Last, the direct factor against *Xoo* invasion in *Xa3/Xa26*-mediated resistance may mainly be the accumulation of phytoalexins in plants with a *japonica* background. *Xa4*-initated defense response to *Xoo* infection is associated with both an accumulation of the phytoalexins molilactone A and sakruranetin and a strengthening of the cell wall through promotion of the expression of cellulose synthesis genes *CesA*s and suppression of the expression of cell wall expansin genes *EXPA*s (Hu et al. 2017). *Xa3/Xa26*-mediated resistance to *Xoo* is also associated with accumulation of molilactone A and sakruranetin in *japonica* rice lines (Fig. 5), but it is only associated with accumulation of sakruranetin not molilactone A in an *indica* rice line compared to susceptible controls (Liu et al. 2012). Previous studies have revealed that JA-Ile is indispensable for sakuranetin production and required in part for momilactone production in rice (Riemann et al. 2013; Shimizu et al. 2013). In this study, JA-Ile and phytoalexin levels in transgenic line Rb49, which overexpresses *Xa3/Xa26*, are significantly higher ($P < 0.05$) than those in near-isogenic line MD1 after *Xoo* infection, respectively. These higher levels may contribute to Rb49 having a higher resistance level than MD1. All these results suggest that accumulation of both molilactone A and sakruranetin may explain *Xa3/Xa26* functioning better in a *japonica* background than in an *indica* background.

In addition, the dosage effect of *Xa3/Xa26* may be associated with accumulation of more phytoalexins in the rice–*Xoo* interaction.

Rice resistance to *Xoo* regulated by other genes is also reported to be associated with suppressed expression of *EXPA*s (Ding et al. 2008; Fu et al. 2011). However, the expression of *EXPA*s was not obviously suppressed in *Xa3/Xa26*-mediated resistance compared to the susceptible control (Supplemental Fig. 2). Furthermore, activation of *CesA*s was only detected during early infection of the resistance rice lines. These results suggest that reinforcement of the cell wall may not be the major contributor against *Xoo* infection in *Xa3/Xa26*-mediated resistance.

In summary, the present results indicate that the durable resistance gene *Xa3/Xa26* is more appropriate for rice improvement with a *japonica* background by traditional breeding methods. The function of *Xa3/Xa26* is better in combination with the *WRKY45-1* allele with either a *japonica* or *indica* background.

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