Development of Markers for Identification and Maker-Assisted Breeding of Xa7 Gene in Rice (Oryza sativa L.)

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Abstract

Utilization of resistance (*R*) genes to breed resistant cultivars is one of the most effective and economical approach to control rice bacterial blight (BB). *Xa7*, a dominant, broad-spectrum and durable BB-resistant gene, is an ideal gene resource to improve the resistance of rice varieties to bacterial blight, and this well-known gene with important breeding value has been cloned in our recent study. The isolation of *Xa7* will facilitate its application in rice breeding by molecular maker-assisted selection (MAS). In this study, based on the specific sequences in the promoter of *Xa7*, a functional marker, named as MX7, was developed, which can effectively distinguish the dominant BB-resistant *Xa7*, the recessive BB-susceptible *xa7* and the null allele from different rice varieties. Since MX7 is a dominant marker, it can't tell homozygous from heterozygous, a co-dominant marker closely linked to *Xa7*, named as M6, was developed simultaneously. After verified by amplification in numerous rice varieties and sequence alignment in RICE 3K database, it is proved that marker M6 is co-segregated with the *Xa7* locus. In addition, the effectiveness and accuracy of the two markers were further validated by two F_2 populations. Finally, the designed makers were effectively applied in MAS breeding to improve the BB-resistance of a susceptible variety. This study not only provides reliable functional markers for the identification of *Xa7* gene in different rice materials, but also will contribute to the application of *Xa7* gene in marker-assisted selection to breed rice varieties with durable disease resistance.

Introduction

Bacterial blight (BB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most destructive diseases of rice in the world, and causes serious yield losses (Mew 1987). Since pesticides is expensive and contaminates environment, the most economical and environmental friendly approach to control BB is the utilization of resistance (*R*) genes to breed resistant varieties (Rao et al. 2002). To date, at least 46 genes conferring host resistance against various *Xoo* races have been identified (Chen et al. 2020), and sixteen of them have been cloned successfully (Chen et al. 2021). Among those *R* genes, *Xa4* was the first to be widely used in modern rice varieties since the late 1960s, especially with the widespread cultivation of high-yielding rice varieties, such as TN1 and IR8, which caused BB to become a major threat to rice production (Khush 1995). Since then, breeding rice varieties with resistance to BB was extremely valued, and with the identification and cloning of new *R* genes, as well as the development of available DNA markers for corresponding genes, rice varieties pyramided with different *R* genes by marker-assisted selection (MAS) have been successfully applied in BB-resistant breeding (Perumalsamy et al. 2010; Bharani et al. 2010; Suh et al. 2013). In fact, with the application of *Xa3*, *Xa4*, *Xa21* and other resistance genes in rice breeding, bacterial blight had almost disappeared for a short time in China (Zhang 2009). However, large-scale and long-term cultivation of varieties carrying a single *R* gene resulted in a significant shift in pathogen race frequency, and eventually led to the breakdown of resistance in these cultivars (Rao et al. 2002; Jiang et al. 2020). For example, the most widely deployed BB resistance gene, *Xa4*, which had been effectively used in rice breeding to control BB for nearly 20 years, but subsequent studies confirmed that its disease resistance has already broke down (Vera-Cruz et al. 2000; Quibod et al. 2019). An effective way to delay the breakdown of BB resistance is to pyramid multiple *R* genes in a single variety, especially application of *R* genes with durable disease resistance ability.

Xa7 is a dominant, broad-spectrum and durable BB-resistant gene (Vera-Cruz et al. 2000; White et al. 2009; Zhang et al. 2015). This gene was originally identified from Bangladesh rice variety DV85 (Sidhu et al. 1978),

and then by backcrossing with an IRRI rice variety IR24, *Xa7* was introduced into IRBB7 (Ogawa et al. 1991). Extraordinarily, according to the results of many years' field trials, the resistance of *Xa7* was proved to be more durable than *Xa4* and *Xa10* (Vera-Cruz et al. 2000). Up to now, *Xa7* is considered to be the most durable resistance gene to bacterial blight. The durable resistance ability of *Xa7* is closely related to the specificity of its cognate avirulence genes, both *avrXa7* and *pthXo3*, which are also virulence genes maintaining toxicity of *Xoo* and are vital for their own survival, so mutations of *avrXa7* or *pthXo3* risk to be eliminated by natural selection, and this special relationship can help to limit the loss of resistance of *Xa7* (Chen et al. 2021; Luo et al. 2021; Vera-Cruz et al. 2000). More interestingly, *Xa7*-containing rice varieties are more effective at high temperatures, whereas other *R* genes are less effective (Webb et al. 2010; Cohen et al. 2017; Dossa et al. 2020). In the context of global warming, *Xa7* is an ideal *R* gene for breeding durable disease resistant varieties.

Based on the excellent characteristics of *Xa7*, mapping based cloning of this gene has been focused for decades by researchers. As early as 1995, *Xa7* was initially located at 107.5-cM on the current RGP map (Kaji and Ogawa 1995). Subsequently, *Xa7* had been reported to be finely mapped into a 2.7-cM region between marker M1 and M3 on rice chromosome 6, and highly linked with marker M5 speculatively (Porter et al. 2003). And then, *Xa7* was further mapped into a 118-kb region flanking with markers GDSSR02 and RM20593 (Chen et al. 2008). After a-decade of hard works, we successfully cloned this gene from a Chinese rice variety Zhen-hui 084 (Chen et al. 2021). Our results showed that *Xa7* is a novel executor *R* gene, it was induced by the corresponding transcription activator-like effectors (TALEs), and the induction was faster and higher under high temperature. In addition, it carries a putative effector binding elements (EBEs) in its promoter (EBE_{AvrXa7}), which are recognized by its corresponding avirulence genes, and the EBE sequence is essential for its BB resistance in rice (Chen et al. 2021). Based on the cloning and specific sequences analysis of *Xa7*, here we reported the development and assessment of a functional marker for identification of *Xa7* in different rice varieties, and the design and application of a co-dominant marker co-segregated with *Xa7* for homozygous analysis of the *Xa7* locus. By tested in multiple rice varieties and segregation populations, as well as the collinearity analysis between molecular markers and the *Xa7* gene locus by sequence alignment in RICE 3K database, we proved that the developed functional marker can accurately identify whether there is *Xa7* gene in different rice varieties, and the combination utilization of the designed functional marker and co-dominant marker is helpful to efficiently application of *Xa7* in MAS breeding.

Material And Methods

Plant materials

Rice variety Zhen-hui 084 is a *Xa7*-containing variety, with durable and broad-spectrum resistance to rice bacterial blight disease. Rice variety Chen-hui 448 doesn't contain the *Xa7* locus, while rice variety Lao-zao-gu carrying the *xa7* allele, both of them is susceptible to *Xoo* strain PXO86. The seeds of the above rice varieties were sowed in the field. At the booting stage, the stamens of Chen-hui 448 or Lao-zao-gu were emasculated artificially by scissors and crossed with the pollen of Zhen-hui 084, and their F_1 plants were self-crossed to develop the corresponding F_2 populations. All of the other rice varieties used in this study were presented by the Chinese National Rice Research Institute (Hangzhou, China).

Evaluation of bacterial blight resistance

Ten *Xoo* races were used in this study to evaluate the bacterial blight resistance of rice plants, and all of them were obtained from Chinese Academy of Agricultural Sciences (Beijing, China). The strains were cultured on agar media containing 20 g of sucrose, 5 g of peptone, 0.5 g $Ca(NO_3)_2$, 0.43 g Na_2HPO_4, and 0.05 g $FeSO_4$ per liter, and allowed to grow at 28°C for 2 to 3 days. The bacterial colony was suspended in sterile distilled water at an optical density of OD_{600} = 0.5 and immediately used for inoculation. The 60-day-old rice plants were inoculated by using leaf-clipping method (Kauffman et al. 1973). Two weeks later, the lesion length on the cut leaves were measured for evaluation of BB-resistant level (Yin et al. 2000).

DNA extraction and PCR amplification

Total genomic DNA was extracted from rice leaves using Panaud et al. (1996) method. GoTaq® Master Mix (Promega, USA) was used for PCR amplification with 10–20 ng genomic DNA and a suitable concentration of primers according to the manufacturer's instructions. For marker the functional MX7, three primers were used and equivalently mixed. The forward primer was named as MX7-FP: 5'-AATATATAACCCCCCCCCCCCCAG-3', the intermediate primer was named as MX7-MP: 5'-ATGGCGGCCGCTGATCATCC-3', and the reverse primer was named as MX7-RP: 5'-TTAATTGCCACCGATGAGGTAATCC-3'. For the co-dominant marker M6, primers pair are named as M6-FP: 5'-GGGAGAAATTGGCCCCAGTTAGAGAA-3' and M6-RP: 5'-GCATGTCTGTGTCGATTCGTCCGTACGA-3'. The primers pair of the marker M5 used here was reported by Porter et al. (2003). PCR process was performed as: (1) pre-denatured at 94 ^{o}C for 5 min; (2) denatured at 94 ^{o}C for 30 s, annealed at a temperature according to the markers (M6 at 60 ^{o}C; MX7 at 62 ^{o}C; M5 at 65 ^{o}C) for 30 s and extended at 72 ^{o}C for 60 s, followed by 35 cycles; (3) finally extended at 72 ^{o}C for more 5 min. The PCR products were examined via 1.5% agarose-gel electrophoresis and stained with ethidium bromide (EB).

Bioinformatic analysis

The alignment of different sequences was carried out by the software named as ClustalX 2.0, and the output of the results was modified by GENEDOC 2.7. The CDS sequences of *Xa7* and the amplified sequences of maker M6 were used for sequences similarity analysis. Due to the miss of the *Xa7* locus in the reference genomes (both Nipponbare and R498), so sequences alignment were performed with the scaffolds of each cultivar in the RFGB database (http://www.rmbreeding.cn/Blast; Wang et al. 2018). In presenting the sequence alignment results, only the varieties with 100% consistent coding region of *Xa7* were listed.

Evaluation of the major agronomic traits

Field trials were tested in the summer season, 30-day-old seedlings of the BC_5F_4 plants derived from the backcross between Zhen-hui 084 and Cheng-hui 448, as well as both the parents were transplanted in the field at 15×25 cm spacing in south China. The experiments were carried out following a randomized complete block design (RCBD) with three replications. The agronomic traits were measured following the standard evaluation system described by Xuan et al. (2019). Ten plants from each entry were recorded as one data replication, including plant height (cm), panicle length (cm), flag-leaf length and width (cm), panicles number per plant, panicle length (cm), grains number per panicle, seed-setting rate (%), grain length and width (mm), and the 1000-grain weight (g). Statistical analysis was performed with independent samples by using *t*-test for significance between the plants of Zhe-kang 1 and Cheng-hui 448.

Results

Design of molecular markers for identification of Xa7 gene

According to our previous results, the nucleotide sequence of the *Xa7* gene is absolutely novel and is not widely existed in rice germplasms (Chen et al. 2021). Therefore, based on the specific coding sequence (CDS) of *Xa7*, markers directly located in this region could be used for functional identification of this gene in major rice varieties. However, in some susceptible varieties, we also found a completely identical CDS as *Xa7*, but with variations in its promoter (with an 11-bp fragment insertion and a G to T substitution in the EBE_{AvrXa7} region), and in order to distinguish it from the dominant BB-resistant gene *Xa7*, this type of allele was designated as *xa7* (Chen et al. 2021; Fig. 1A). On this basis, a functional PCR marker containing three primers, named as MX7, was designed (Fig. 1A). The forward primer (MX7-FP) was located in the EBE_{AvrXa7} region, and the middle primer (MX7-MP) was located in the CDS from the start codon, while the reverse primer was located in the CDS before the stop codon (Fig. 1A). The three primers mentioned above were mixed and used for a single PCR amplification. Theoretically, in rice varieties containing the BB-resistant gene *Xa7*, a 482-bp fragment (partial promoter + whole CDS) and a 342-bp (only the whole CDS) fragment will be simultaneously amplified, while in rice varieties carrying the *xa7* allele, because of the variation in the EBE_{AvrXa7} region was unmatched by the forward primer during annealing, only a 342-bp fragment would be amplified. In addition, in varieties absence of the *Xa7* locus, no sequence could be amplified at all.

As the functional marker MX7 is more of a dominant marker for most rice varieties, and cannot effectively distinguish the homozygote and heterozygote of *Xa7*. Therefore, it would be better to develop a co-dominant marker highly linked or co-segregated with *Xa7*. Previously, according to the reference genome of Nipponbare, we finally mapped *Xa7* into a 51-kb region flanking with markers M5 and M10 (Chen et al. 2021), which are more closely linked to *Xa7* than the reported markers used in the fine mapping of *Xa7* (Porter et al. 2003; Chen et al. 2008; Fig. 1B). Actually, several co-dominant molecular markers of *Xa7* have been developed and applied in rice breeding, among them, M5 is more closely linked to *Xa7* (Porter et al. 2003; Chen et al. 2008; Perez et al. 2008; Zhang et al. 2010; Huang et al. 2012; Yap et al. 2016; Mi et al. 2018). However, the distance between M5 and *Xa7* is still 92.3-kb on its true physical map (Fig. 2B), so that it may not be able to co-segregated with *Xa7.* By sequence alignment, fortunately, a 227-bp fragment insertions and deletions (indels) was found between IRBB7 and IR24 in the region only 16.6-kb away from *Xa7* (Fig. 1C, D). Based on this indels, a co-dominant molecular marker, named as M6, was designed, the predictive fragments amplified from IRBB7 and IR24 are 306-bp and 531-bp, respectively (Fig. 1D).

Verification of the designed markers in different types of rice varieties

Eighteen rice varieties were used for verification of the designed markers, including six varieties containing the dominant resistant gene *Xa7*, seven varieties carrying the recessive susceptible gene *xa7*, and the other five varieties without this locus at all (Chen et al. 2021; Fig. 2). The amplification results of MX7 were consistent with the expected results, the 342-bp and 482-bp fragments (partial promoter + whole CDS) were simultaneously amplified from all of the *Xa7* containing varieties, but only the 342-bp fragment (whole CDS) was amplified from all of the *xa7* carrying varieties, and no fragment was amplified from all of the null-allele varieties (Fig. 2A). These results indicated that MX7 is a dominant marker, and could be used to distinguish *Xa7* from *xa7* in rice cultivars. For marker M6, the 306-bp fragment was amplified from the varieties containing

Xa7 or *xa7*, while the 531-bp fragment was amplified from the null-allele varieties (Fig. 2B). As a comparison, marker M5 was also analyzed, the amplification result of marker M5 was similar to that of marker M6, except for the polymorphisms of M5 were less conserved in the *Xa7* null-allele varieties (Fig. 2C). These results suggested that both M5 and M6 have co-dominant polymorphisms between *Xa7* (or *xa7*) and the null allele, but cannot distinguish *Xa7* from *xa7*.

To verify whether marker MX7, M6 and M5 could be widely used for identification of *Xa7* in rice varieties, a total of 167 Chinese rice varieties were assessed by the three markers and inoculated with PXO86, a *Xoo* strain containing the corresponding avirulence gene *avrXa7* and can be used in the identification of *Xa7* (Table S1). The identification results of MX7 showed that no varieties were detected containing *Xa7*, and twenty-one varieties were detected carrying the *xa7* allele, while the other 146 varieties were detected as the null-allele of the *Xa7* locus. The bacterial blight inoculation results were consistent with MX7, none of the 167 varieties were highly resistant to PXO86. For maker M6, an about 306-bp fragment were amplified from all of the *xa7* carrying varieties, while an 531-bp fragment were amplified from all of the null-allele varieties (Table S1). However, compared to M6, there is no such good consistent polymorphism of marker M5 in these varieties (Table S1). These results were further confirmed by varieties from other countries, as shown in Table 1, maker MX7 can accurately identify the *Xa7* gene in varieties from different countries, and in terms of the linkage with the *Xa7* locus, maker M6 is more precise than M5.

Table 1
Information of the varieties with *Xa7*, *xa7* or null allele.

No.	Variety name	Origin	[a] M5	[b] M6	[c] MX7	[d] PXO86	[e]Lesion length (cm)
1	IRBB7	Philippines	+	+	*Xa7*	HR	0.6 ± 0.29
2	DV85	Bangladesh	+	+	*Xa7*	HR	0.4 ± 0.15
3	DV86	Bangladesh	+	+	*Xa7*	HR	0.5 ± 0.23
4	AUS 242	Bangladesh	+	+	*Xa7*	HR	0.3 ± 0.06
5	AUS 299	Bangladesh	+	+	*Xa7*	HR	0.4 ± 0.08
6	AUS 308	Bangladesh	+	+	*Xa7*	HR	0.4 ± 0.07
7	Zhen-hui 084	China	+	+	*Xa7*	HR	0.5 ± 0.12
8	Lao-zao-gu	China	+	+	*xa7*	S	29.2 ± 2.50
9	ARC 10100	India	+	+	*xa7*	S	37.1 ± 2.37
10	DANGAR	India	+	+	*xa7*	S	26.6 ± 1.99
11	BARI SUTAR	India	+	+	*xa7*	S	33.2 ± 5.78
12	MHARAKA	Kenya	+	+	*xa7*	S	34.4 ± 2.22
13	MANSARA DHAN	Nepal	+	+	*xa7*	S	31.3 ± 0.92
14	MOTIA	Pakistan	+	+	*xa7*	S	28.0 ± 3.67
15	CHAO HAI	Laos	-	+	*xa7*	S	30.4 ± 2.47
16	KHAO SIM	Thailand	-	+	*xa7*	S	28.0 ± 1.54
17	Lu-cai-hao	China	-	+	*xa7*	S	24.7 ± 1.68
18	Jin-bao-yin	China	-	+	*xa7*	S	24.1 ± 1.82
19	Xiang-wan-xian 3	China	+	-	Null	S	27.7 ± 1.06
20	Zhen-xian 232	China	+	-	Null	S	27.0 ± 1.39
21	Hong-ai-nuo	China	+	-	Null	S	24.9 ± 2.09
22	Shan-jiu-gu	China	+	-	Null	S	22.2 ± 3.56
23	76 – 1	China	-	-	Null	MR	5.2 ± 1.17

[a] Genotypes of the marker M5 in the varieties. '+' refers to a IRBB7-like fragment and '-' refers to other types fragments. [b] Genotypes of the marker M6 in the varieties. '+' refers to the 306-bp fragment and '-' refers to the 531-bp fragment. [c] Genotypes of the alleles identified by the functional marker MX7. *Xa7* refers to the BB-resistant dominant gene, *xa7* refers to the recessive susceptible allele with an identical CDS of *Xa7* but variations in the promoter, and null refers to an allele without the *Xa7* CDS and its promoter. [d] High resistant (HR), medium resistant (MR), medium susceptible (MS) or susceptible (S) phenotype of the varieties inoculated by the *Xoo* strain PXO86; [e] the lesion length of infected leaves 2 weeks after inoculated with POX86. The value of traits is measured as mean ± S

No.	Variety name	Origin	[a] M5	[b] M6	[c] MX7	[d] PXO86	[e]Lesion length (cm)
24	IR24	Philippines	-	-	Null	S	27.2 ± 1.73
25	Zhong-hua 11	China	-	-	Null	S	25.9 ± 0.52
26	Nipponbare	Japan	-	-	Null	MS	7.8 ± 0.48
27	9311	China	-	-	Null	S	22.3 ± 0.64
28	Cheng-hui 448	China	-	-	Null	S	26.9 ± 0.45

[a] Genotypes of the marker M5 in the varieties. '+' refers to a IRBB7-like fragment and '-' refers to other types fragments. [b] Genotypes of the marker M6 in the varieties. '+' refers to the 306-bp fragment and '-' refers to the 531-bp fragment. [c] Genotypes of the alleles identified by the functional marker MX7. *Xa7* refers to the BB-resistant dominant gene, *xa7* refers to the recessive susceptible allele with an identical CDS of *Xa7* but variations in the promoter, and null refers to an allele without the *Xa7* CDS and its promoter. [d] High resistant (HR), medium resistant (MR), medium susceptible (MS) or susceptible (S) phenotype of the varieties inoculated by the *Xoo* strain PXO86; [e] the lesion length of infected leaves 2 weeks after inoculated with POX86. The value of traits is measured as mean ± S

In order to further analyze whether marker M6 is co-segregated with *Xa7*/*xa7* allele, the sequences of *Xa7*/*xa7* and marker M6 were used for the collinear analysis in the database of 3010 cultivated rice varieties. As listed in Table S2, total 410 rice varieties were found to contain alleles with a 100% identity of the *Xa7* CDS, but were not sure to be *Xa7* or *xa7* due to the poor quality of sequencing in the EBE region (poly C or G). Meanwhile, there are 401 varieties containing an identical sequence with the 306-bp fragment amplified from IRBB7, and the sequence similarity of the other 9 varieties are also more than or equal to 95% (Table S2). In addition, no *Xa7* or *xa7* allele was found in more than 2500 rice varieties, and most of them containing an identical sequence with the 531-bp fragment (data not shown). The above results proved that maker M6 is co-segregated with the *Xa7* locus.

The information of some representative varieties contained the *Xa7*, *xa7* or null alleles were listed in Table 1, including genotypes of the markers and phenotypes of the BB resistance to PXO86. Our results indicated that the marker MX7 could be used for functional identification of *Xa7* in rice varieties, and the marker M6 is co-segregated with the *Xa7* locus better than marker M5. Thus, combination of the functional marker MX7 and the co-dominant marker M6 together can be effectively used for the identification and marker-assisted breeding of *Xa7*.

Verification of the designed markers in segregated populations

In addition, in order to evaluate the accuracy of the designed primers in segregated populations, the *Xa7*-containing variety Zhen-hui 084 was crossed with the null-allele variety Chen-hui 448, and the F_1 progenies were self-crossed to build the F_2 segregated population. The F_2 plants were individually amplified with marker MX7, and inoculated with PXO86. The 342-bp and 482-bp bands of MX7 were both amplified in the resistant plants, but were not amplified in the susceptible plants (Fig. 3A). Although marker MX7 could functionally identify *Xa7* from the resistant plants, obviously, it could not distinguish homozygote and heterozygote of the *Xa7* locus (Fig. 3A). Therefore, marker M6 was used for further analysis of these plants, and it clearly displayed the difference among dominant homozygote, heterozygote and recessive homozygote of the *Xa7* locus

(Fig. 3A). An F_2 segregated population derived from the cross between the *Xa7*-containing variety Zhen-hui 084 and the *xa7*-carrying variety Lao-zao-gu was also built, subsequently analyzed by both the designed markers and POX86. In this situation, M6 was useless due to no polymorphism between the two parents, but MX7 could still identify the resistant plants by the specific 482-bp band in spite of the 342-bp bands existed in all of the F_2 plants (Fig. 3B). The above results indicate that for the identification of *Xa7* in rice varieties, only the functional maker is credible, and there are a large number of false positives when utilization of molecular markers closely linked to or co-segregated with the *Xa7* locus. Unfortunately, a large number of previous studies have used these unreliable closely linked molecular markers to identify whether there is *Xa7* gene in rice varieties (Deo et al. 2013; Majumder et al. 2020; Prasannakumar et al. 2021; Ullah et al. 2012).

Application of the designed markers in MAS breeding

The rice variety Cheng-hui 448, a Chinese *indica* restorer line with wide compatibility and good qualities, was bred from a cross between American rice Lemont and Chinese restorer Line 871028 (Ren et al. 1999). This variety is highly resistant to *Magnaporthe grisea*, but is generally susceptible to *Xoo* (Fig. 5). To improve its BB resistance, Cheng-hui 448 was selected as a recurrent parent and backcrossed with the *Xa7*-containing variety Zhen-hui 084 (Fig. 4). At that time, as the *Xa7* gene has not been isolated, all the plants of BC_1F_1, BC_2F_1, BC_3F_1, BC_4F_1 and BC_5F_1 were amplified by marker M6, and the PXO86-resistant plants with the 306-bp fragment of M6 were further backcrossed with Cheng-hui 448 (Fig. 4). And then, the homozygous BC_5F_1 plants selected by M6 were further self-crossed for three generations to get the BC_5F_4 plants, which were finally identified by the functional marker MX7 after we cloned *Xa7*. Finally, through three times of background selection, the BC_5F_4 plants was regarded as the near isogenic lines containing *Xa7* gene, we named it as Zhe-kang 1. All of the Zhe-kang 1 plants could amplified the 342-bp and 482-bp bands of MX7 (data not shown) and displayed a broad-spectrum of BB resistance (Fig. 5). Moreover, there was no significant difference of the mainly agronomic traits between Cheng-hui 448 and the improved near isogenic line Zhe-kang 1 (Table 2).

Table 2
The major agronomic performance of the improved line Zhe-kang 1 and the recurrent parent Cheng-hui 448.

Variety	Plant height (cm)	Flag-leaf length (cm)	Flag-leaf width (cm)	No. of tillers per plant	Panicle length (cm)	No. of grains per panicle	Spikelet fertility (%)	Grain length (mm)	Grain width (mm)	1000-grain weight (g)
Cheng-hui 448	105.72 ± 0.97	29.92 ± 0.19	1.58 ± 0.12	10.17 ± 0.75	28.18 ± 0.17	144.67 ± 8.07	77.74 ± 1.75	10.39 ± 0.38	2.76 ± 0.07	25.53 ± 0.25
Zhe-kang 1	106.54 ± 0.42	30.28 ± 0.47	1.68 ± 0.08	10.10 ± 0.63	28.10 ± 0.14	147.40 ± 8.44	77.32 ± 1.51	10.47 ± 0.46	2.80 ± 0.12	25.68 ± 0.18

Data shown as Mean ± SD. Statistical analysis was performed by a two-tailed *t*-test for paired samples, and there were no significant difference in all of the tested traits.

Discussion

Host-plant resistance is a cost-effective and environmentally safe approach to reduce yield loss caused by rice bacterial blight disease. However, the acquisition of host-plant resistance depends on the identification and application of resistance genes. In fact, among the 46 identified BB-resistance genes, only few of them have been widely used in rice breeding, such as *Xa3*、*Xa4*、*Xa7*、*Xa21* and *Xa23* (Zhang et al. 2009; Mi et al. 2018). The main reason is that some genes have poor resistance, or narrow-spectrum, and part of them are recessive genes, which limited them to be widely used in hybridization breeding. At the same time, whether the resistance of the *R* genes can be sustained also needs to be considered. Many varieties with *Xa4* have been cultivated widely in Asia to control BB disease; the large-scale and long-term cultivation of those varieties carrying *Xa4* resulted in significant shifts in the race frequency of *Xoo*, and eventually led to the decline of resistance to bacterial blight. In this study, according to the resistance characteristics of *Xa7*, such as broad-spectrum (Ogawa et al. 1991), long-lasting (Vera-Cruz et al. 2000) and heat-tolerant (Webb et al. 2010), we selected the newly cloned *Xa7* as the gene resource to develop broad-spectrum and durable-resistance rice variety. However, although the resistance of *Xa7* has been proved to be more durable, whether it can escape the migration of pathogenic population needs to be further studied.

Transgenic technology and molecular maker-assisted selection are the main ways to utilize disease resistance genes in breeding. Due to the evaluation and supervision of transgenic crops are very strict, MAS is still the main way for resistance improvement in rice (Wong et al. 2016; Jin et al. 2019). Therefore, it is of great significance to development molecular markers that can be used in MAS breeding. In the present study, based on the specific sequence of the *Xa7* gene, a functional marker of *Xa7* and a co-dominant marker that co-segregated with *Xa7* were developed. The main reason of designing two molecular markers for breeding application of *Xa7* is the particularity of its own alleles in different rice cultivars. Unlike most of the other *R* genes, there are three types of the *Xa7* locus in rice varieties, which are *Xa7*-containing-resistant type, *xa7*-containing susceptible type, and the null-allele susceptible type. Sequence difference between *Xa7*-containing-resistant type and *xa7*-containing susceptible type only existed in the EBE region of the *Xa7* promoter, and whether this difference is the same in different rice varieties is still difficult to identify (Chen et al. 2021), therefore, it is not available to design a co-dominant marker at this site. Moreover, as shown in Table S2, only a small proportion of rice materials containing the *Xa7* or *xa7* alleles, while more than 2500 rice varieties belong to the null-allele susceptible type. As homozygote and heterozygote in MAS breeding cannot be distinguished only by the functional marker MX7, hence, combination of the functional marker MX7 and the co-dominant marker M6 together will be more effectively for the identification and marker-assisted breeding of *Xa7*. For identification of *Xa7*, it is worth noting that utilization of molecular markers closely linked to or co-segregated with the *Xa7* locus is not reliable.

The application of molecular markers makes it more convenient to transfer resistance genes into a variety. However, the accuracy and validity of the designed molecular markers should be evaluated before be used in breeding. In this study, in order to estimate the efficiency of the designed molecular markers, firstly we analyzed the physical distance between different markers and the target gene *Xa7* (Fig. 1B and Fig. 1C). Subsequently, utilization of different rice cultivars from both China (Table S1) and all over the world (Table 1), the polymorphisms of the designed markers in those varieties were identified. As exhibited in Fig. 2 and Table 1, consistent with the result of *Xoo* inoculation, the functional marker MX7 can specifically identify *Xa7* gene from the *Xa7*-containing rice varieties, while the co-dominant marker M6 showed a better linkage with the *Xa7* locus when compared to previously used marker M5. And then, the above results were further confirmed by a more

large population based on the high throughput data information of the 3010 rice accessions (Table S2). In addition, the designed makers were verified in practical breeding to improve the bacterial blight resistance of a BB-susceptible variety Cheng-hui 448. The analysis of major agronomic traits revealed that the introduction of *Xa7* gene enhanced the BB-resistance of Cheng-hui 448, but did not impact the yield and other main agronomic traits (Fig. 5). Thus, the makers developed in this study can not only be used to identify *Xa7* gene from different germplasm, but also guide the application of *Xa7* in molecular marker-assisted breeding.

Conclusions

In this study, the functional marker MX7 for identification of *Xa7* and the co-dominant marker M6 that co-segregated with the *Xa7* locus were developed. And combination of the functional marker MX7 and the co-dominant marker M6 together can be effectively used for the identification and marker-assisted breeding of *Xa7*.

Declarations

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Authors' contributionsJBM and XFC designed and supervised the experiments. PCL, LM and LMH performed the experiments. YLX and YTZ contributed to MAS breeding of Zhe-kang 1. QQ, XMZ and DLZ provided rice varieties and revised the manuscript. CPL, LM and XFC wrote the manuscript with input from all other co-authors. All the authors have read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest All the authors declare that they have no competing interests.

Ethical approval We declare that the present experiments comply with the ethical standards of

the country in which they were performed.

Data Availability All data related to this study have been submitted.

Animal Research (Ethics) Not applicable in this study.

Consent to Participate (Ethics) Not applicable in this study.

Consent to Publish (Ethics) Not applicable in this study.

Plant Reproducibility The plants used in this study can be reproduced.

Clinical Trials Registration Not applicable in this study.

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Figures

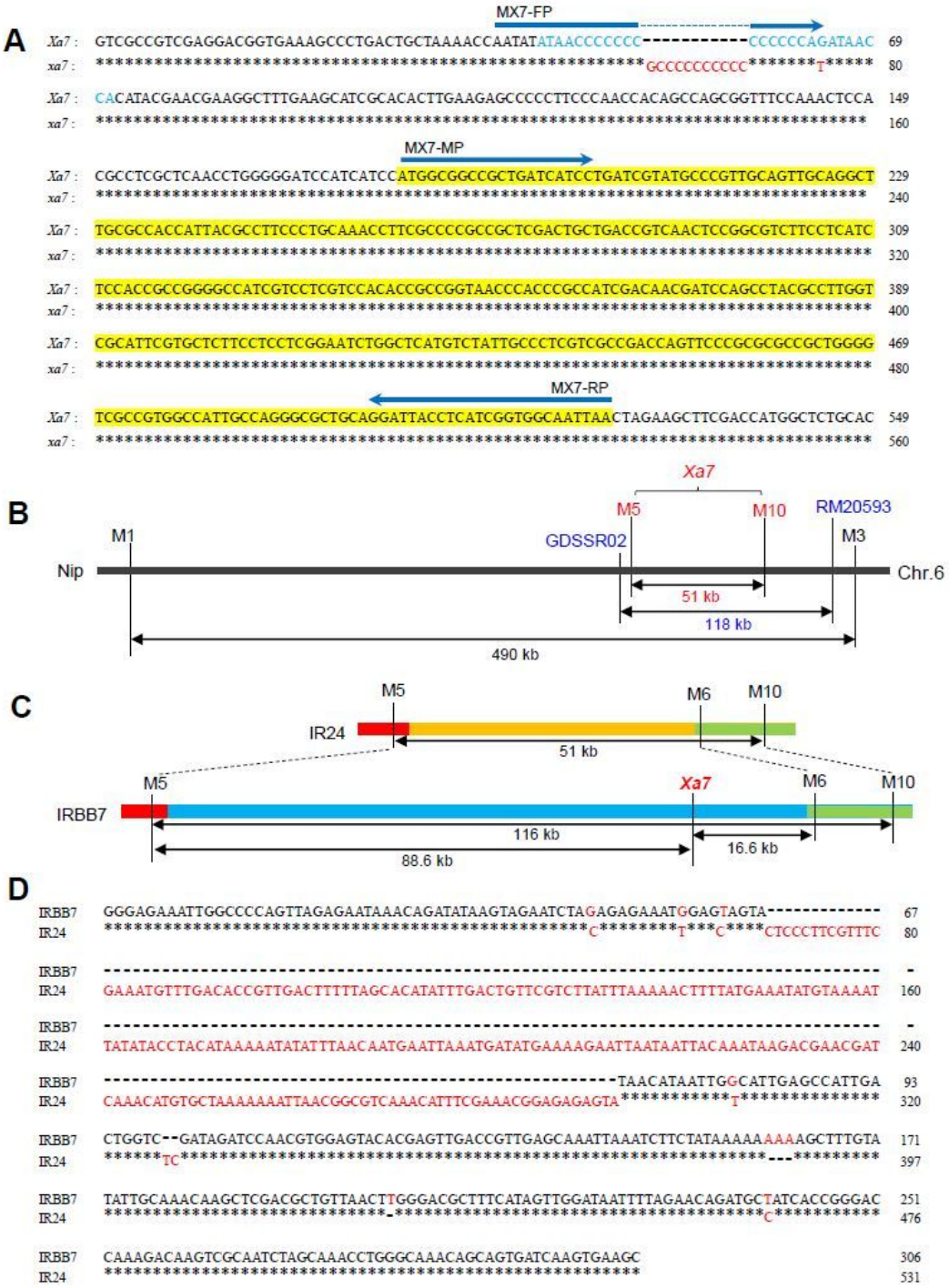

Figure 1

Design makers for identification of the Xa7 alleles. A Sequence alignment between Xa7 and xa7. Nucleotides consistent with Xa7 are labeled as '*'. The 342-bp CDS of Xa7 is highlighted with yellow shading. The EBEAvrXa7 sequence in the promoter of Xa7 is colored in blue, while the 11-bp insertion and a G to T substitution in the xa7 allele are colored in red. The primers locations of marker MX7 are exhibited with arrow. B Physical map of Xa7 and the markers used in fine mapping based on the Nipponbare genome. The markers M1, M3 and M5 were reported by Porter et al. (2003), the markers GDSSR02 and RM20593 were reported by Chen et al. (2008), and the markers M10 were designed by ourselves (Chen et al. 2021). C Physical distance

and sequence homology of IRBB7 and IR24 in the Xa7-mapping region. The regions with same color, red on left board and green on right board, are homologous. The regions with different colors indicate no homology. D Sequence alignment of the M6 fragments amplified from IRBB7 and IR24. Identical nucleotides are shown as '*', and the deletion or insertion nucleotides are shown as dotted lines.

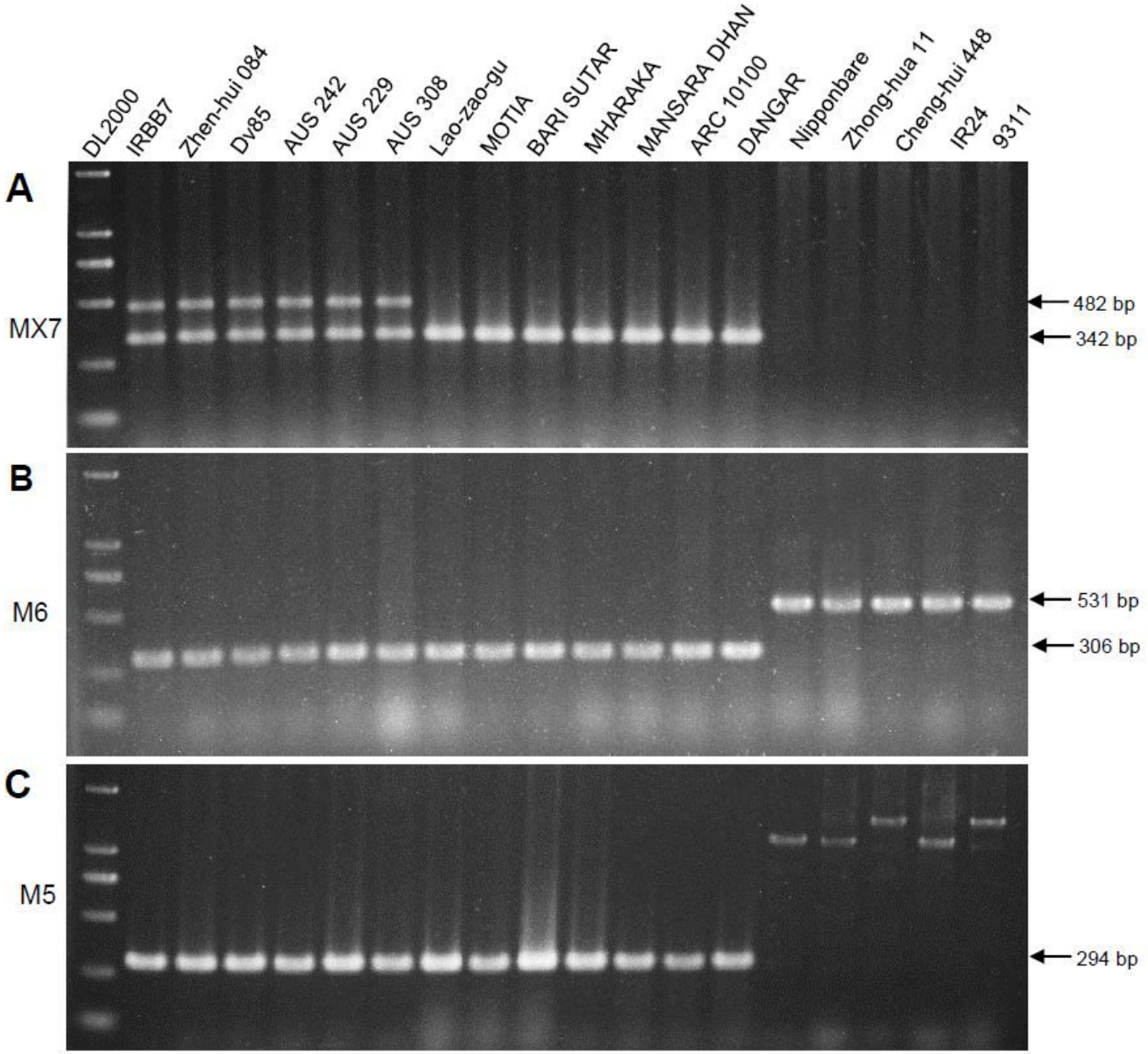

Figure 2

Genotyping of marker MX7, M6 and M5 in rice varieties carrying different types of the Xa7 alleles. A Genotyping of the functional marker MX7 at the Xa7 locus. B Genotyping of the co-dominant marker M6 closely linked to Xa7. C Genotyping of the co-dominant marker M5 linked to Xa7. Rice variety IRBB7, Zhen-hui 084, DV85, AUS 242, AUS 229 and AUS 308 containing the dominant resistant gene Xa7. Lao-zao-gu, MOTIA, BARI SUTAR, MHARAKA, MANSARA DHAN, ARC 10100 and DANGAR carrying the recessive susceptible allele xa7.

Nipponbare, Zhong-hua 11, Cheng-hui 448, IR24 and 9311 own the null allele of Xa7/xa7. DL2000 is the DNA marker, and the objective fragments amplified by corresponding markers were indicated with arrow and followed by the sizes.

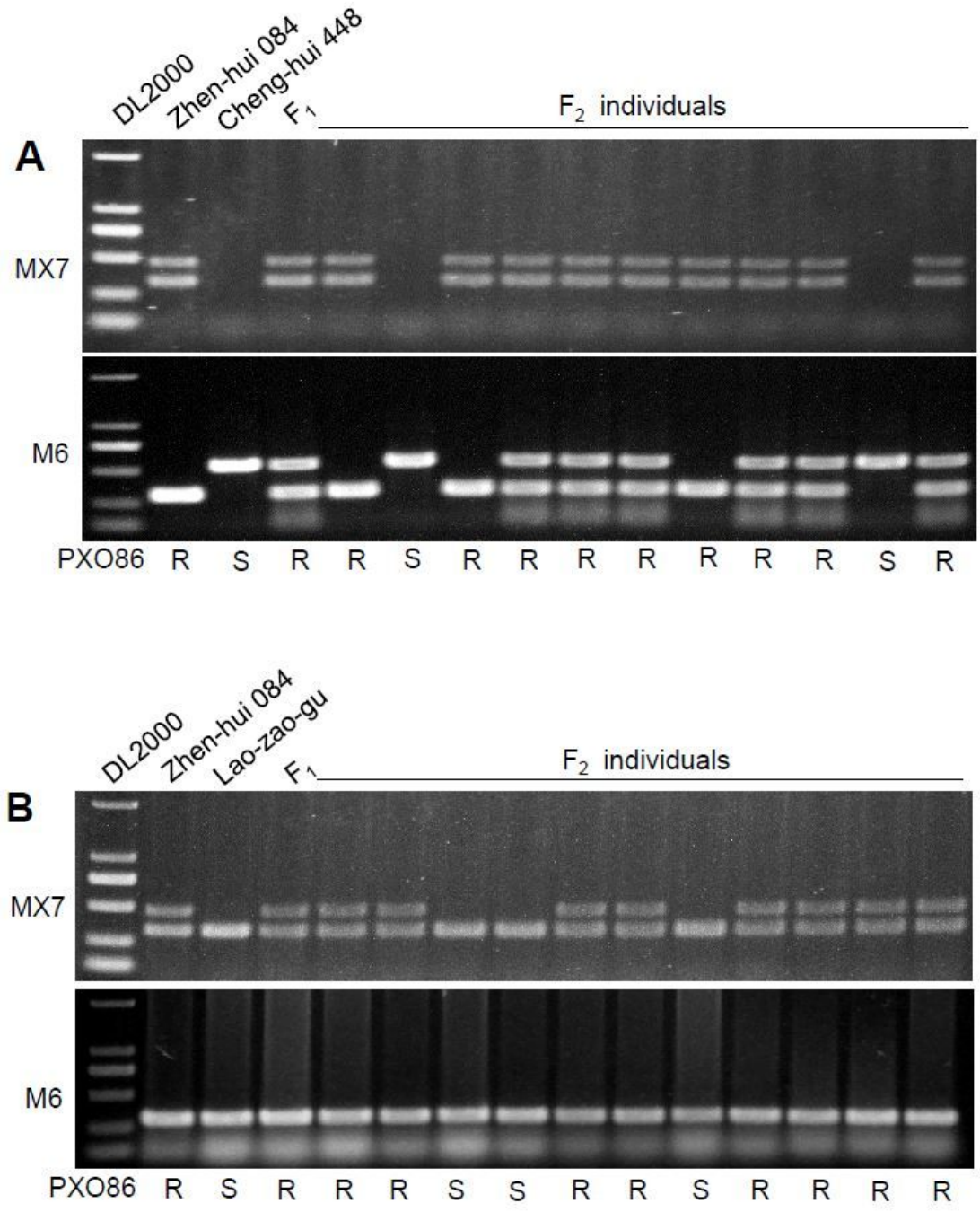

Figure 3

Genotyping of marker MX7 and M6 in segregated populations. A Genotyping in F2 population derived from the cross between Zhen-hui 084 (Xa7) and Cheng-hui 448 (null allele). B Genotyping in F2 population derived from the cross between Zhen-hui 084 (Xa7) and Lao-zao-gu (xa7). The parents, F1 and F2 plants were inoculated with Xoo strain PXO86 to test the BB-resistant (R) or susceptible (S) phenotype. DL2000 is the DNA marker.

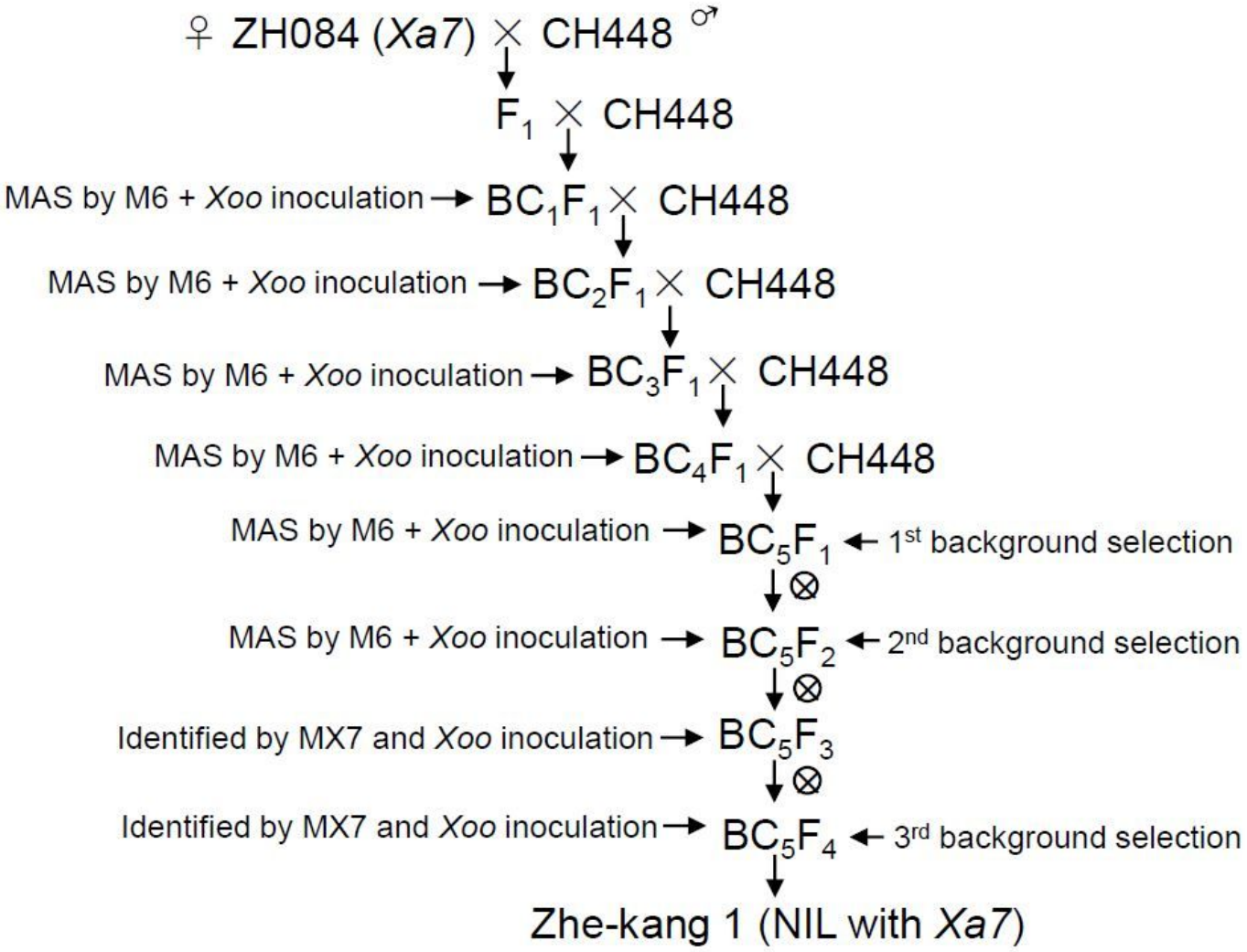

Figure 4

Schematic diagram of marker-assisted breeding of Zhe-kang 1. The recurrent parent Cheng-hui 448 (carrying null-allele of Xa7) was backcrossed with Zhen-hui 084 (the donor of the BB-resistant Xa7). The progenies were amplified with M6 or MX7 for marker-assisted selection of Xa7 in each generation. Individual plant (with excellent agronomic traits) selected from BC5F4 was designated as Zhe-kang 1, a near isogenic line carrying the homozygous alleles of Xa7. The progenies in each generation were inoculated with Xoo strain PXO86 for evaluation of resistance to bacterial blight.

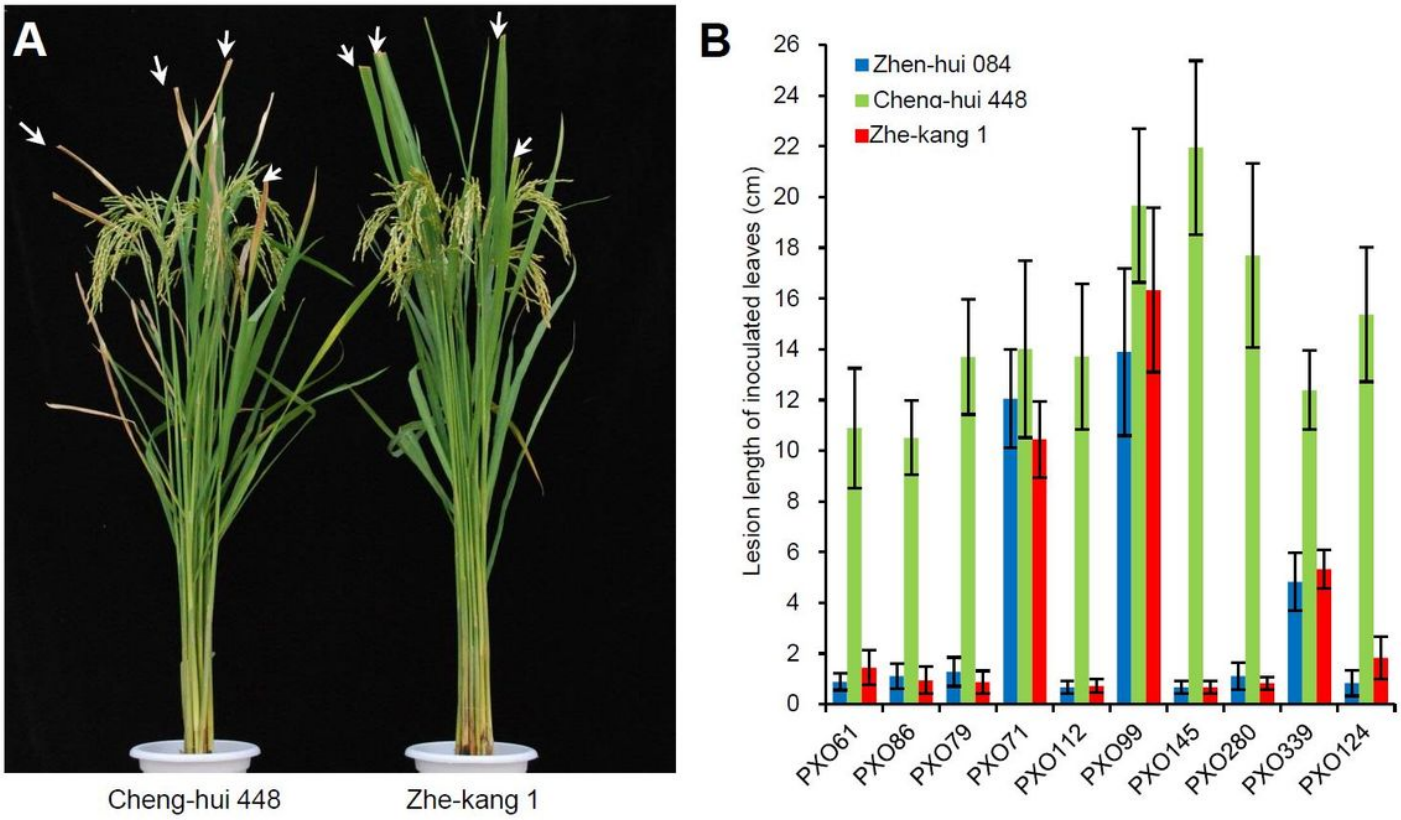

Figure 5

Resistance evaluation of the improved near isogenic line Zhe-kang 1 to Xoo strains. A Phenotypes of individuals inoculated with Xoo strain PXO86 for two weeks. Arrows indicate the sites on leaves inoculated with PXO86 by clipping method (Kauffman et al. 1973). A serious bacterial blight disease symptom displayed in the leaves of Cheng-hui 448, but not in the near isogenic line Zhe-kang 1. B Resistance spectrum of Zheng-hui 084, Cheng-hui 448 and the improved near isogenic line Zhe-kang 1. Individuals were inoculated with 10 Xoo races identified by IRRI; the lesion length of inoculated leaves was measured 2 weeks after infection. Bars refer to the standard errors.

Supplementary Files

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- TableS1.xlsx