Marker-Assisted Introgression of a Bacterial Blight (BB) Resistance Gene, *Xa21*, in Rice RD47 and Evaluation of BB Resistance in F_1 Hybrid (RD47 x IRBB21)

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ABSTRACT

The RD47 rice is a popular variety widely grown in the lower-north area of Thailand, but it has a major drawback as it is susceptible to the Bacterial Blight (BB) caused by *Xanthomonas oryzae* pv. oyzae (*Xoo*). This BB is a very destructive disease leading to severe yield losses. The dominant and broad spectrum *Xa21* BB resistance gene has been widely used in breeding programs. RD47 used as a recipient parent was crossed with the *Xa21* carrying donor parent IBB21 in order to provide BB resistance. Marker assisted selection (MAS), using both the pTA248 (closely linked to the *Xa21* gene) and XA21A (based on the *Xa21* gene sequence) markers, was used to select the F_1 and BC_1F_1 progenies bearing *Xa21*. Finally, the F_1 progenies were proved to be BB resistant by a *Xoo* infection test. Our results demonstrate that a single copy of *Xa21* in the heterozygous *Xa21* genotype was sufficient to give *Xa21*-mediated BB resistance.

Keywords: Bacterial blight, Marker-assisted selection, Rice, *Xa21*, *Xanthomonas*, *Xoo*

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Introduction

Rice (*Oryza sativa*) is one of the most important food crops. Bacterial Blight (BB) caused by *Xanthomonas oryzae* pv. oyzae, is a very destructive disease leading to severe yield losses in rice production worldwide (Narayanan *et al*., 2004). Breeding for resistant varieties is thought to be the most economic and effective means to control the disease. (Yan-chang *et al*, 2004)

More than 35 BB resistance genes have been identified in cultivated and wild rice (Nino-Liu *et al*., 2006; Guo *et al*., 2010; Miao *et al*., 2010). Of these BB resistance genes, *Xa21* discovered in wild rice, *O. longestaminata*, is a dominant gene which confers a broad-spectrum resistance to most of the *Xoo* strains (Khush *et al*., 1989, 1990; Ikeda *et al*., 1990). The *Xa21* gene was introgressed to *O. sativa* IR24 through backcross. The near isogenic line IRBB21 containing the *Xa21* gene with the background of IR24 exhibited resistance to all tested *Xoo* strains from the Philippines and India (Ikeda *et al*., 1990). Therefore, the *Xa21* gene has been widely used in rice breeding programs especially through introgression using molecular markers. For example, a Shuangyou 4 1 8 3 variety susceptible to BB was improved to BB resistance by introgression of *Xa21* gene using PCR-based markers, pTA248 (closely linked to *Xa21* gene) and XA21 (based on the *Xa21* gene sequence), for selection (Yan-chang *et al*., 2004). Moreover, three resistant varieties (BC_3F_2 KMR3, PRR78 and Mahsuri) were developed to resist BB using Npb181 (*Xa4*), RG136 (*xa13*), pTA248 (*Xa21*), and RM 122 (*xa5*) markers (Shanti *et al*., 2010).

pTA248 and Xa21 markers are co-dominant markers which can identify and distinguish the *Xa21* genotypes between homozygous resistance, heterozygous resistance and homozygous susceptible. The pTA248 marker generates the PCR products approximately 925 bp, 730 and 925 bp and 730 bp for homozygous resistance, heterozygous resistance and homozygous susceptible, respectively. The Xa21 marker produces the PCR products approximately 1.3 kb, 1.2 and 1.3 kb and 1.2 kb for homozygous resistance, heterozygous resistance and homozygous susceptible, respectively (Yan-chang *et al*., 2004).

Rice cultivar RD47, non-glutinous and photoperiod-insensitive, was developed by the Phitsanulok rice research center (PRRC) and has been one of popular cultivars grown in lower Northern Thailand. It has a high productivity and good seed quality. RD47 cultivar is rather resistant to brown planthopper (BPH) and blast disease but susceptible to bacterial blight (BB) disease (Bureau of Rice Research and Development: Rice Department, Thailand). To improve the RD47 for resistance to BB by introgression of the *Xa21* gene, the marker assisted selection can be used to generate a BB resistance RD47 isogenic line through backcross breeding.

Objective of the study

In this study, we aimed to improve the RD47 rice variety by introducing the *Xa21* BB resistance gene from IRBB21 variety through conventional breeding coupled with marker-assisted selection (MAS), and assess the BB resistance of the F_1 hybrid (RD x IRBB21) via a *Xoo* inoculation test.

Materials and Methods

Plant material

IRBB21 rice, developed from an IR24 near isogenic line, carries the *Xa21* gene. It is resistant to

BB, and used as a donor (male) parent. RD47 rice variety carries null *Xa21* gene. It is susceptible to BB, and used as a recipient (female) parent. These two varieties were cultivated on soil with individual pots and grown outdoor at Naresuan University, Phitsanulok.

Cross pollination

At the appropriate flowering stage (25% of the panicle emerged from the flag leaf), the female spikelet was carefully emasculated from 3 to 6 pm. At 10-11.30 am on the following day, they were pollinated with the male pollen using the hand-dusting technique. The pollinated spikelet was covered with paper bag for 30 days, and the seeds were then harvested.

Breeding strategy

The breeding strategy used to produce F_1 and BC_1F_1 lines shows in (Fig. 1).

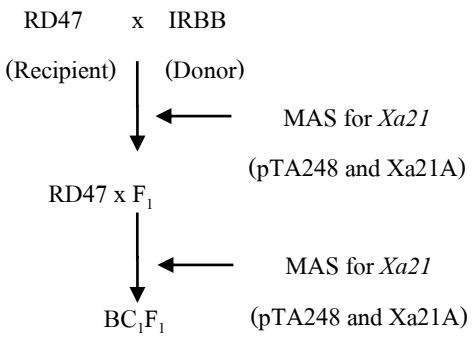

Figure 1 Schematic view of the breeding strategy with marker-assisted selection for *Xa21* gene.

The genotypes of the F_1 and BC_1F_1 plants were determined by using PCR with the pTA248 and Xa21A markers.

The pTA248 marker is closely linked to *Xa21* gene (0-1 cM). The primer sequences are pTA248-Forward (5'-AGACGCGGAAGGGTGGTTCCCGGA-3') and pTA248-Reverse (5'-AGACGCGGTGTAATCGAAAGATGAAA-3'). The pTA248 primers gave PCR products of approximately 925 bp and 730 bp in *Xa21* and null *Xa21* genotypes respectively.

The Xa21A marker is a dominant marker designed from published sequence of the *Xa21* gene (accession number U72723). The primers are Xa21A-Forward (5'-GGGAAGTGCCAACCATTGGTG-3') and Xa21A-Reverse (5'-CCTCCATCAGTTCATGTAGAAG-3'). The Xa21A primers gave a PCR product of approximately 1152 bp only in the *Xa21* genotype.

Thermo Scientific Phire Plant Direct PCR Master Mix® was used, according to the manufacturer protocol. Briefly, 1mm diameter leaf samples were cut, and crushed in 20µl of dilution buffer. The mixture (1µl) was used as DNA template in the PCR solution (H_2O 8 µl, 2X Phire Direct PCR master mix 10 µl, Primer Mix 7.5 µM each).

PCR conditions were set as follow: initial pre-denaturation at 98 °C for 5 min, and then 40 cycles of denaturation 98 °C for 5 sec, annealing 60 °C (for pTA248) or 63 °C (for Xa21A) for 10 sec, extension 72 °C for 22 sec. The amplified PCR products were detected by electrophoresis on a 1% Agarose gel.

Xoo

A PLS'008 *Xoo* strain isolated from a Phitsanulok2 infected plant growing in the Phitsanulok province was provided by the PRRC. This *Xoo* strain was cultivated on NA culture medium and incubated at 28 °C for 48 h. Inoculation solution was prepared by resuspending the bacteria in sterilized distilled water. Bacterial concentration was

adjusted to 10^9 cfu/ml and a few drops of Tween20 were added.

Evaluation of BB resistance

Sixty-day old F_1 plants (maximum tillering stage) were tested for BB resistance by the clipping method inoculation test (Kauffman *et al.*, 1973). Briefly, three selected flag leaves on each plant were cut in length 1-2 cm by scissors previously dipped in the *Xoo* inoculation solution. Twenty-one days after inoculation, lesion length on cut leaves were measured by scoring according to IRRI (Table1). The RD47 and IRBB21 were used as susceptible and resistant control respectively.

Table 1 IRRI scoring system used to evaluate breeding line for BB resistance

Description	Lesion Length (cm)
Resistance (R)	0-5
Moderately Resistance (MR)	>5-10
Moderately Susceptible (MS)	>10-15
Susceptible S	>15

Results

Breeding and MAS

The results of the F_1 plants genotyping with the pTA248 marker are shown in the Fig. 2. A PCR product of approximately 730 bp has been amplified for the RD47 recipient parent while an approximately 925 bp was amplified for the IRBB21 donor parent. As for the F_1 progenies, both the approximately 730 bp and the 925 bp PCR products have been amplified for F_1 individuals. This shows that all the F_1 plants tested were heterozygous carrying a single copy of the *Xa21* gene. These heterozygous F_1 plants were then backcrossed with RD47 plants. The resulting BC_1F_1 plants were screened for the presence of the *Xa21* gene using the pTA248 marker (Fig. 3a). For all of the BC_1F_1 plants a PCR product of approximately 730 bp, corresponding to the null *Xa21* genotype was amplified. However, only 3 of the 7 BC_1F_1 plants showed the approximately 730 and 925 bp PCR products corresponding to the heterozygous *Xa21* genotype. As the pTA248 marker is only closely linked to the *Xa21* gene, the XA21A primers which allow for the amplification of a 1152 bp fragment of the *Xa21* gene, were used to confirm the presence of this gene in the plants (Fig. 3b). As expected, the 1152 bp fragment was amplified only in the 3 plants that had previously shown a polymorphic heterozygous profile with the pTA248 marker.

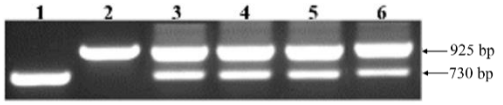

Figure 2 Genotyping of the F_1 plants using the pTA248 marker. Lanes 1, RD47, 2, IRBB21, 3-6 F_1 RD47x IRBB21 hybrid rice plants (F_1-1, F_1-2, F_1-3 and F_1-4).

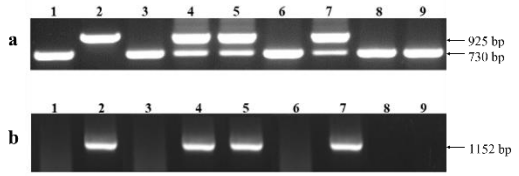

Figure 3 Genotyping of the BC_1F_1 plants using the pTA248 and Xa21A markers. 3a, The *Xa21* genotype screening with the pTA248 marker, 3b, The *Xa21* genotype screening with the Xa21A marker. Lanes 1, RD47, 2, IRBB21, 3-9, BC_1F_1 plants (BC_1F_1-1, BC_1F_1-2, BC_1F_1-3, BC_1F_1-4, BC_1F_1-5 and BC_1F_1-6).

Evaluation of BB resistance

Artificial inoculation was performed with the PLS'008 *Xoo* strain to evaluate the resistance of the heterozygous F_1 progenies. The parental plants, RD47 and IRBB21 were used as susceptible and resistant control respectively. Infected leaves were collected at 21 days after inoculation (Fig. 4), and the BB lesion length (LL) were measured and scored (Table 2).

The recipient parent, RD47 displayed an average lesion length of 10 cm and was thus scored as moderately susceptible (MS) while the donor parent IRBB21, with an average lesion length of 0.2 cm was classified as resistance (R). All the four F_1 hybrid line F_1-1, F_1-2, F_1-3, and F_1-4 with average lesion length of 0.2 cm, 0.5 cm, 0.3 cm and 0.5 cm, respectively also displayed a high level of resistance.

Discussion

Genotyping of the F_1 hybrid RD47 x IRBB21 showed that they were all heterozygous carrying a single copy of *Xa21*. The result is in accordance with the breeding theory which predicts that crossing a homozygous dominant parent with a homozygous recessive parent results in 100% heterozygous progenies. However, as the breeding process is prone to mistakes, checking the F_1 genotype is an important step in order to eliminate any fake F_1 plants resulting from undesired self-pollination. Concerning the backcrosses, the breeding theory predicts that the BC_1F_1 plants will consist of 50% homozygous susceptibility (null *Xa21*) and 50% heterozygous resistance (*Xa21*/-). Genotyping of seven BC_1F_1, showing four homozygous plants and three heterozygous plants are indeed very close to the expected backcross ratio (50:50). Genotyping of the BC_1F_1 plants using pTA248 and XA21A markers identified BC_1F_1-2, BC_1F_1-3 and BC_1F_1-5 as heterozygous plants carrying the *Xa21* gene. In fact that was expected as the pTA248 is closely linked to the *Xa21* gene (>1 CM) (Yan-chang *et al*., 2004). Most of the *Xa21* introgression breeding programs only use the pTA248 marker for the MAS (Huang *et al*., 2003; Yan-chang *et al*., 2004, 2012; Sundaram *et al*., 2008), as it is more efficient for screening. However, we still decided to use the XA21A marker to confirm that the *Xa21* gene really existed in our selected plants.

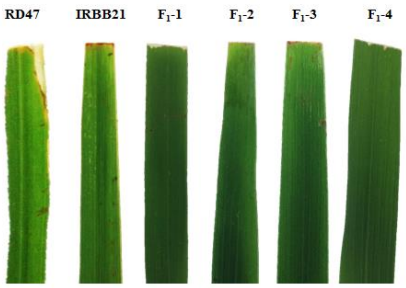

Figure 4 BB Lesions on the leaves of RD47, IRBB21 and F_1 plants at 21 days after *Xoo* inoculation.

Table 2 Lesion length measurement and scoring of resistance in RD47, IRBB21 and F_1 leaves at 21 days after *Xoo* inoculation.

	Average lesion length* (cm)	scoring of resistance
RD47	10.2	MS
IRBB21	0.2	R
F_1-1	0.2	R
F_1-2	0.5	R
F_1-3	0.3	R
F_1-4	0.5	R

*Calculated from duplication of the inoculation test.

In regard to the *Xoo* infection test, the RD47 plants showed large lesions corresponding to a MS phenotype and thus assessed for the efficiency of the infection. In the meantime, heterozygous F_1 plants only showed very small lesions corresponding to a R phenotype. In fact the heterozygous F_1 plants carrying only one copy of the dominant *Xa21* gene have reached a resistance level similar to the one of the homozygous IRBB21.

Conclusion

We have successfully bred the BB susceptible RD47 rice variety with the BB resistant IRBB21 variety and obtained F_1 and BC_1F_1 progenies carrying the *Xa21* gene. Moreover, we were able to prove that the heterozygous F_1 plants bearing the *Xa21* gene were effectively highly resistant to the BB disease.

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