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Full Paper

Identification of microsatellite markers (SSR) linked to a new bacterial blight resistance gene *xa33*(t) in rice cultivar 'Ba7'

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Abstract: This study attempts to identify a new source of bacterial blight (BB) resistance gene and microsatellite makers (SSR) linked to it. A total number of 139 F_2 progenies generated from a cross between the resistant donor 'Ba7'and 'Pin Kaset' were developed and used for this study. A Thai *Xoo* isolate, TXO16, collected from Phitsanulok province, was used to evaluate the resistance reaction in the F_2 population. The segregation ratio of resistance (R) and susceptibility (S) was statistically fitted to 1R:3S model indicating single recessive gene segregation. Twenty F_2 individuals consisting of 10 resistant and 10 susceptible plants were chosen for DNA analysis. Sixty-two polymorphic markers covering all rice chromosomes were used to identify the location and linked markers of the resistance gene. Four SSR markers, viz. RM30, RM7243, RM5509 and RM400, located on the long arm of rice chromosome 6, could clearly discriminate between resistant and susceptible phenotypes, and 161 $BC_2F_{2:3}$ individuals carrying BB resistance gene were developed through MAS using these SSR markers. This population was inoculated with TXO16 to validate and confirm the location of the gene and linked markers. The segregation ratio was statistically fitted to 1R:3S model confirming a recessive nature of the gene action in this germplasm. Phenotypic-genotypic association including five additional markers suggested that RM20590 was tightly linked to this resistance gene (R^2=59.12 %). The BB phenotype was controlled by a recessive gene with incomplete dominance of susceptible allele providing intermediate resistance to *Xoo* pathogen in heterozygotes. The location of the gene was in the vicinity of a dominant gene, *Xa7*, which was previously reported. However, the resistance gene identified here was different from *Xa7* because of the different nature of gene action. Consequently, this gene was tentatively designated as *xa33*(t). The resistance gene from rice cultivar 'Ba7' and the closely linked markers found in this study will be useful for rice breeders as a source to improve BB resistance through MAS in rice breeding programs.

Keywords: bacterial blight, rice, SSR marker, *Xanthomonas oryzae* pv. *oryzae*, *xa33*(t)

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Introduction

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the most destructive diseases in rice-producing areas in Thailand and it is especially prevalent in irrigated and rainfed lowland rice growing areas. BB was first characterised in Fukuoka (Japan) in 1884 [1]. In Thailand, the damage of rice crop from BB was first reported in 1963 in Pathum Thani province [2]. BB disease can cause yield loss typically ranging from 20-30%, but in severely cases it can cause as high as 50% yield reduction [1]. Control measures for BB include cultural practices, chemical control, biological control, disease forecasting, and most importantly, host genetic resistance. Since the most effective chemical control is not yet available, the utilisation of resistant varieties carrying resistance genes has been considered to be the most effective way to control the disease [3]. Most researchers are interested in utilising BB-resistant varieties, and this goal is certainly achievable provided that an easy strategy to identify resistance genes is available.

At present, identification, cloning and functional analysis of a gene can be performed more rapidly. Up to date, more than 40 disease-resistant genes have been identified in dicot and monocot plants [4]. In the case of BB resistance, more than 30 BB resistance genes have been identified in cultivated rice and the wild relatives [3, 5-6]. Eleven of them are recessive resistance genes (*xa5, -xa5(t), xa8, xa13, xa15, xa19, xa20*, *xa24*, *xa28*, *xa31* and *xa32*) [3, 6-7], while six of them are cloned (*Xa1, xa5*, *xa13*, *Xa21 Xa26* and *Xa27*) [8-13]. Among all BB resistance genes, three of them were reported and mapped on rice chromosome 6. The first one, *Xa7*, is a dominant resistance gene originally identified in rice cultivars 'DV85' and 'DV87'. The second BB resistance gene, *Xa27*, identified in wild rice *O. minuta*, has also been mapped to the same region as *Xa7* [14-15], and the resistance gene has been cloned afterward [13, 16]. The last one, a recessive BB resistance gene *xa32*, identified in wild rice *O. barthii*, is located on the short arm of the same chromosome [6].

The majority of BB resistance genes were identified in rice *O. sativa* ssp. *indica* and wild rice *O. longistaminata*, *O. rufipogon*, *O. minuta* and *O. officinalis*, while some of them were identified in *O. sativa* ssp. *japonica* [16-17]. Most of these genes follow the classic gene-for-gene concept for the race-specific interaction between rice and *Xoo* [18]. Avirulent gene in bacteria exhibits the specificity for resistance gene in the rice plant. Some resistance genes are effective only in adult plants, while others are effective at all stages of growth. Some genes confer resistance to a broad spectrum of *Xoo* races, whereas others do so against only one or a few races. This observation could be influenced by particular geographical locations [3]. The developmental control of disease resistance has been observed in other plant-pathogen systems. Several rice resistance genes are expressed at the highest level only at the adult stage [19-20]. *Xa21*-mediated resistance increases progressively from susceptible juvenile stage to full resistance at the later adult stage, while *Xa7* shows broad resistance only in adult plants [21]. However, the effective gene at all growth stages appears to be *xa5* gene as it can confer resistance and exhibit a broad spectrum of resistance to *Xoo* isolates throughout Asia except India and Nepal [22].

The first step towards rice improvement via marker-based selection and map-based cloning of the resistance genes is the identification of molecular markers that are tightly linked to the genes of interest. Recent advances in molecular marker technology have made it easier to identify and introgress resistance genes to desired genetic backgrounds. Several major resistance genes against bacterial blight pathogen have been tagged by restriction fragment length polymorphism (RFLP) and randomly

amplified polymorphic DNA sequence (RAPD) markers [23-25]. In addition, simple sequence repeat (SSR) markers have been extensively used to identify disease resistance genes in rice [26-27]. They provide several advantages over the other two types of markers when applied in a plant breeding program. Markedly, they are based on the polymerase chain reaction (PCR) technique, represent single loci, and can detect high levels of polymorphism.

In this study, we aim at using SSR markers to identify the BB resistance gene in rice cv. 'Ba7' and finding the markers tightly linked to this gene. These markers would be useful for the improvement of BB-resistance rice breeding program through marker-assisted selection (MAS).

Materials and Methods

Plant materials

The *indica* rice cultivar 'Ba7' was used as a BB resistance donor, in a cross with the recurrent parent, 'Pin Kaset' (PK) to develop an F_2 population. The population consisted of 139 progenies that were used as plant materials to identify the genomic location of a BB resistance gene. The backcross breeding and MAS strategies were used to develop the backcross population to validate the linked markers and confirm the location of resistance gene. The F_2 resistant plant was crossed with the recurrent parent to generate 98 BC_1F_1 individuals. DNA markers indentified in the F_2 population were used to select BC_1F_1 plants carrying the resistance gene, and 10 selected BC_1F_1 plants based on desired plant type were then crossed with PK to generate 122 BC_2F_1 individuals. DNA markers were used to identify the 52 BC_2F_1 plants carrying the resistance gene. A heterozygous plant was self-pollinated to produce 838 BC_2F_2. After that, four heterozygous BC_2F_2 plants were self-fertilised to produce 161 $BC_2F_{2:3}$ plants. All these plants were used individually to validate the effect of the resistance gene and the relationship between BB resistant phenotype and linked DNA markers (Figure 1).

Bioassay of BB resistance

A *Xoo* isolate, TXO16, collected from Wang Thong district, Phitsanulok province, Thailand, in 2003 was used in this study for the BB resistance evaluation. This isolate showed an incompatible reaction to 'Ba7' and a compatible reaction to PK. The isolate was grown in peptone sucrose agar medium (5 g peptone, 20 g sucrose and 15 g agar, adjusted to 1 litre with distilled water) for 72 hours at 28°C. The bacterial cells were suspended in sterile water adjusted to 10^9 CFU/ml. TXO16 was assayed for a resistance reaction in F_2 and $BC_2F_{2:3}$ population (Figure 1). BB inoculation was done in the greenhouse using the leaf-clipping method [28]. Resistance reactions were recorded based on the mean of lesion length (LL) of an individual plant. One hundred and thirty-nine F_2 plants were inoculated 30 days after sowing, whereas 161 $BC_2F_{2:3}$ plants were inoculated 60 days after sowing. Three to four fully expanded leaves of each plant were inoculated. LL was measured at 12-14 days after inoculation. Reaction to BB was classified as resistant (R) when the LL was less than or equal to that of the donor parent (Ba7), and as susceptible (S) when it was longer.

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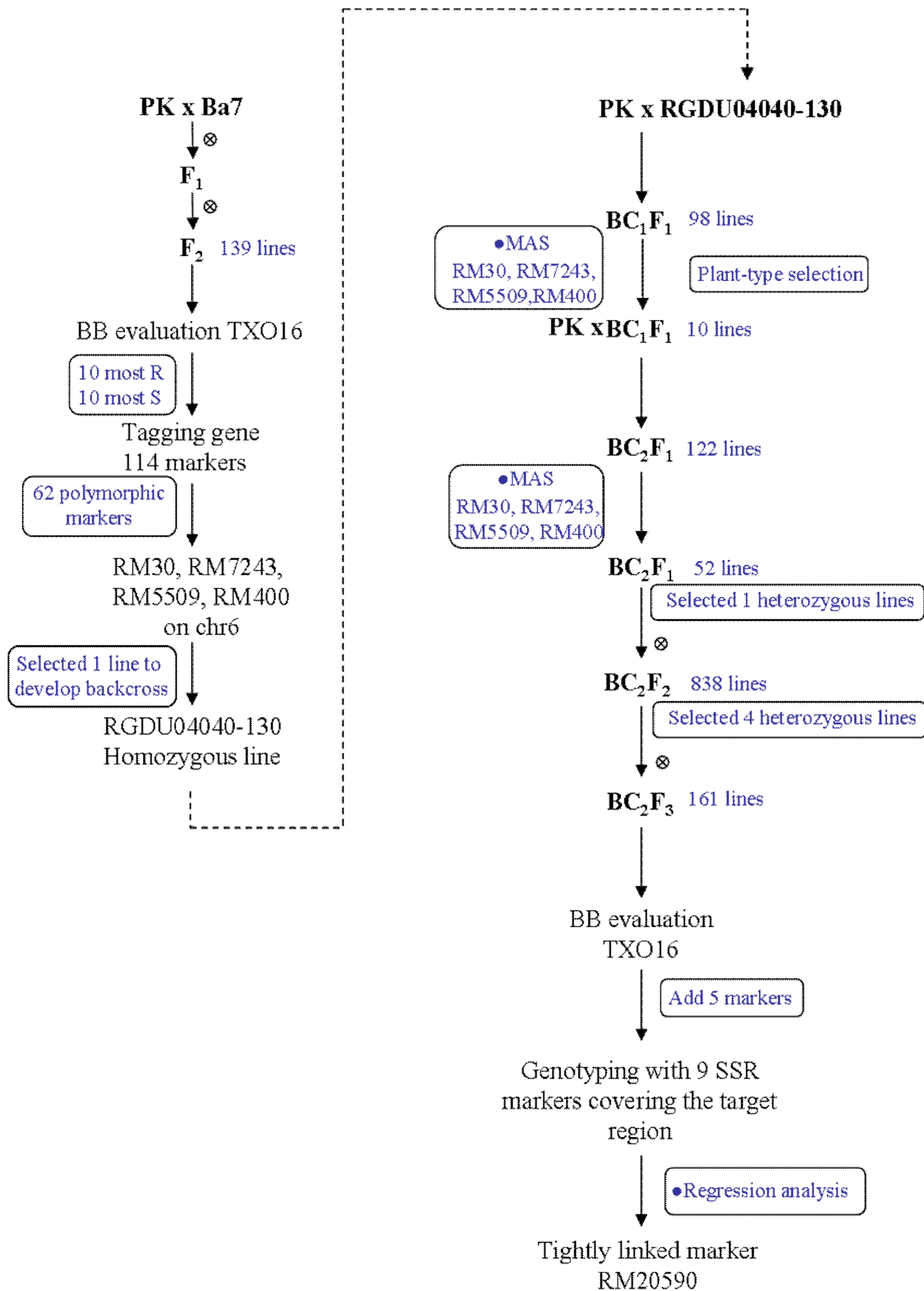

Figure 1. The development of rice lines for identifying SSR markers linked to the resistance gene in rice cultivar 'Ba7'

SSR markers and association analysis

$\underline{F_2}$

One hundred and fourteen rice SSR markers covering the 12 linkage groups were analysed for polymorphism between 'Ba7' and PK. Ten most resistant and susceptible F_2 plants were selected based on their LL and genotypes as identified by 62 polymorphic SSR markers.

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$BC_2F_{2:3}$

Four flanking SSR markers for BB resistance genes, viz. RM30, RM7243, RM5509 and RM400, were used for MAS and genotyping the $BC_2F_{2:3}$ progenies to validate the resistance gene and identify tightly linked markers. Five SSR markers tightly linked to BB resistance genes *xa33*(t), RM20523, RM20536, RM3430, RM20590 and RM340, were added into the $BC_2F_{2:3}$ genotype. These markers were obtained from the public database released by Gramene (http://www.gramene.org/). All of the genetic associations were analysed based on simple linear regression and ANOVA in STATGRAPHIC 2.1 program.

DNA extraction and PCR amplification

The DNA extraction of F_2 and $BC_2F_{2:3}$ population was conducted using the DNA trap® kit (DNA Technology Laboratory). The PCR amplification reactions for SSR markers were carried out with a total volume of 10 µl containing 20 ng of genomic DNA, 0.02 µM of each primer, 0.2 mM each of dNTPs, 2.5 mM $MgCl_2$, 0.2 unit *Taq* polymerase, and 1X PCR buffer. Amplification was performed for 35 cycles (30 sec at 94°C, 30 sec at 55°C and 1 min at 72°C) followed by a final extension of 5 min at 72°C. Amplified products were separated by 4.5% denaturing acrylamide gel electrophoresis and were detected by the silver staining method.

BB resistance reaction patterns between Ba7 and IRBB7

Rice variety 'IRBB7' developed by IRRI was known to carry the dominant gene *Xa7*. It was used to compare BB resistance reaction pattern with 'Ba7'. The *Xa7* and *xa33*(t) were located in the same region on chromosome 6. Sixty-three *Xoo* isolates, collected from major rice growing areas in the north and north-east of Thailand, were used for BB evaluation. The resistance reaction was classified as resistant (R), moderately resistant (MR), moderately susceptible (MS) and highly susceptible (S) when the LL was 0-3 cm, 3.1-6.0 cm, 6.1-9.0 cm and more than 9.0 cm respectively [29].

Results

Phenotypic distributions

Continuous distributions of LL were observed in F_2 and $BC_2F_{2:3}$ population (Figures 2-3). Averages of LL ranged from 0.9 - 2.1 cm and 6.0 - 8.6 cm for 'Ba7' and PK respectively. When the cutoff was based on the mean and standard error of 'Ba7', the numbers of resistant and susceptible F_2 and $BC_2F_{2:3}$ were 37 and 102, and 38 and 124 respectively. These segregation ratios fit well with the expected 1R:3S at χ^2=0.19, p=0.65 and χ^2=0.21, p=0.65 respectively, thereby confirming that the major quantitative trait locus (QTL) for BB resistance in 'Ba7' was governed by a single recessive gene. In our study, inoculations of plants were conducted at two different growth stages, i. e. seedling (30-day-old plants) and tillering (60-day-old plants) stages. It should be noted that LL at the seedling stage was a little bit longer than one at the tillering stage.

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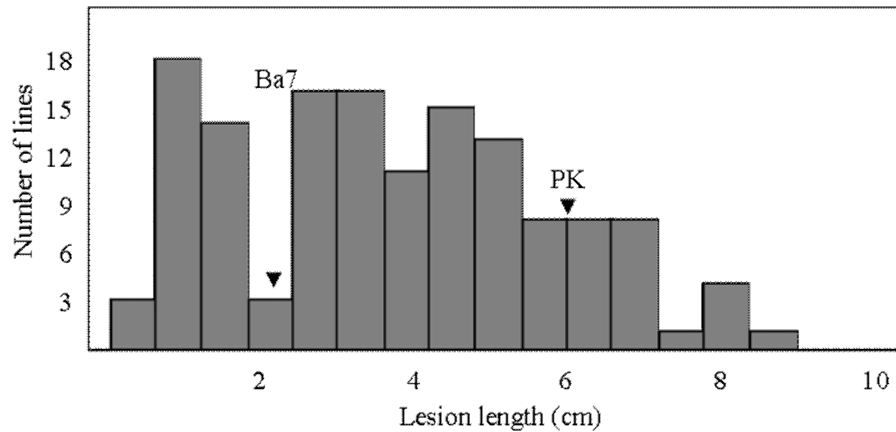

Figure 2. Distribution of LL after inoculation with Thai *Xoo* strain, TXO16, in a sample consisting of 139 individuals from a F_2 population derived from a cross between 'Ba7' and PK. The average LL of 'Ba7' and PK were 2.1 ± 1.4 cm and 6.0 ± 1.6 cm respectively.

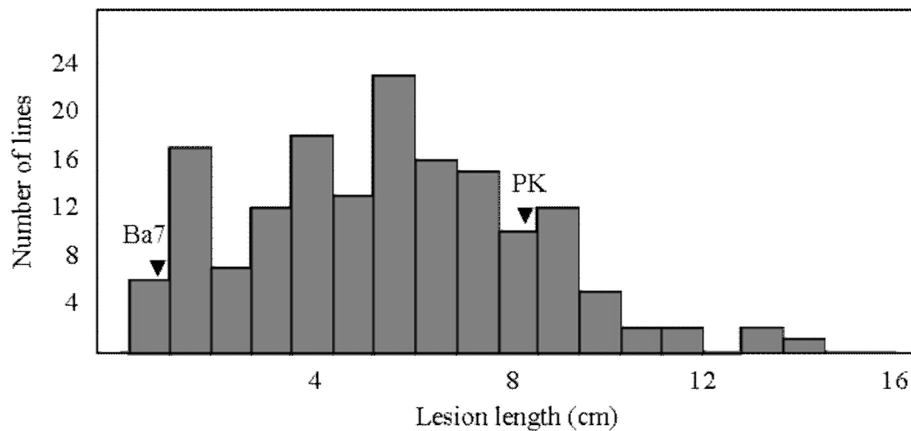

Figure 3. Distribution of LL after inoculation with Thai *Xoo* strain, TXO16, in a sample consisting of 161 individuals from the $BC_2F_{2:3}$ population derived from a cross between 'Ba7' and PK. The average LL of 'Ba7' and PK were 0.9 ± 0.8 cm and 8.6 ± 1.7 cm respectively.

Comparison of BB resistance in Ba7 and IRBB7

Race-specific BB resistance of 'Ba7' and 'IRBB7' were compared at the seedling stage (30 days after sowing) using 63 *Xoo* isolates collected from major rice growing areas in the north and north-east of Thailand (data not shown). 'Ba7' was resistant to 44 isolates, whereas 'IRBB7' was resistant to 41 isolates. Out of 63, 12 isolates showed different patterns or degrees of resistance between 'Ba7' and 'IRBB7' (Table 1). 'Ba7' was resistant to TXO56, while 'IRBB7' was susceptible to TXO56. In contrast, 'Ba7' was susceptible to TB0096 but 'IRBB7' was resistant to TB0096. These results indicated that 'Ba7' and 'IRBB7' were different in their specificity to BB isolates.

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Table 1. Twelve *Xoo* isolates showing different resistance patterns between 'Ba7' and 'IRBB7'

Xoo isolate	Collection area		Resistance pattern	
	Province	Region	Ba7	IRBB7
TB0096	Phitsanulok	North	S	R
TB0304	Chiang Rai	North	S	MS
TB9602	Chiang Mai	North	R	MR
TXO53	Phrae	North	S	MS
TXO55	Chiang Rai	North	MR	S
TXO56	Chiang Rai	North	R	S
TXO103	Ubon Ratchathani	North-east	MS	MR
TXO111	Ubon Ratchathani	North-east	MR	MS
TXO114	Khon Kaen	North-east	MR	R
TXO116	Khon Kaen	North-east	MS	S
TXO121	Udon Thani	North-east	R	MR
TXO122	Udon Thani	North-east	MR	MS

Tagging the major QTL with SSR markers

Out of 114 SSR markers tested for polymorphism, 62 markers revealed clear discrimination between 'Ba7' and PK. These markers were used to identify the genotype of twenty F_2 plants (10 resistant and 10 susceptible plants) and their parents. Four SSR markers, RM30, RM7243, RM5509 and RM400, produced distinguishable band patterns between resistant and susceptible plants as shown in Figure 4. All of them were located on the long arm of rice chromosome 6.

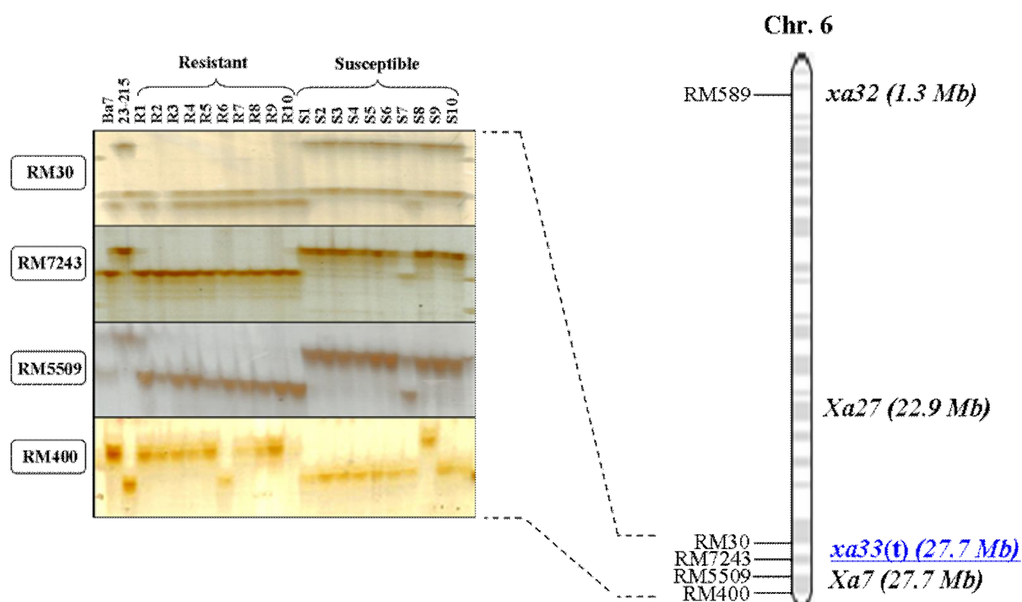

Figure 4. The SSR markers RM30, RM7243, RM5509 and RM400, located on the long arm of chromosome 6, showed distinguishable band patterns of resistant and susceptible F_2 plants and were identified as the possible linked markers to a BB resistance gene in 'Ba7'. These markers were located in the vicinity of reported *Xa*7.

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Identification of the major QTL by phenotype-genotype association

Four selected BC_2F_2 plants that showed heterozygozity via 4 SSR markers mentioned above were self-pollinated to generate 161 $BC_2F_{2:3}$ individuals. These individuals were evaluated for BB resistance reaction and their genotypes were classified by TXO16. RM20523, RM20536, RM3430, RM30, RM7243, RM5509, RM400, RM20590 and RM340 covering this QTL region. Regression analysis confirmed that LL was significantly associated with eight markers designated RM20523, RM20536, RM3430, RM30, RM7243, RM5509, RM20590 and RM400 (27.15-28.43 Mb). Multiple regression analysis indicated that RM20590 and RM5509 were closer to the targeted BB resistance gene than the others. The RM20590 explained 59.12 % of LL variation and appeared to be the closest linked marker in this experiment as shown in Table 2.

Table 2. Phenotype-genotype association analysis using ANOVA and regression analysis in the $BC_2F_{2:3}$ population from the cross between PK and Ba7. The mean of LL was significantly associated with eight SSR markers. (Ba7 = homozygous Ba7, H = heterozygous, and PK = homozygous Pin Kaset.)

Marker	Genome position (Mb)	R^2	Mean of LL (cm)			
			Ba7	H	PK	
RM20523	27.15	58.20**	1.6a	5.1b	8.3c	**
RM20536	27.16	48.88**	1.7a	5.0b	8.0c	**
RM30	27.25	58.20**	1.65a	5.09b	8.34c	**
RM3430	27.43	52.00**	1.94a	5.04b	8.24c	**
RM7243	27.56	57.16**	1.48a	5.11b	8.16c	**
RM5509	27.82	58.33**	1.53a	5.17b	8.31c	**
RM20590	28.01	59.12**	1.49a	5.13b	8.41c	**
RM400	28.43	52.23**	1.60a	5.28b	8.30c	**
RM340	28.59	ns	4.48a	5.27a	5.59a	ns

Notes:
** = significant at 0.01 level
ns = not significant
Means of LL followed by different letters in the same row were significant at $P < 0.01$ by Least Significant Difference (LSD).

Gene action of the major QTL

The LL of $BC_2F_{2:3}$ progenies after inoculation with *Xoo* isolate TXO16 showed a continuous distribution comprising of three phenotypic classes, as seen in Table 2 and Figure 5. Out of 161 $BC_2F_{2:3}$ individuals screened with the closely linked marker RM20590, the results indicated that 27 $BC_2F_{2:3}$ plants were resistant, 99 were moderately resistant, and 35 were susceptible, corresponding to homozygous 'Ba7' alleles (*xa33/xa33*), heterozygous alleles (*Xa33/xa33*), and homozygous PK (*Xa33/Xa33*) alleles respectively. The plants carrying heterozygous alleles (*Xa33/xa33*) exhibited an

intermediate resistance in response to the *Xoo* pathogen, demonstrating that the inheritance of *xa33*(t) is a recessive gene with an incomplete dominance of susceptible allele in gene action.

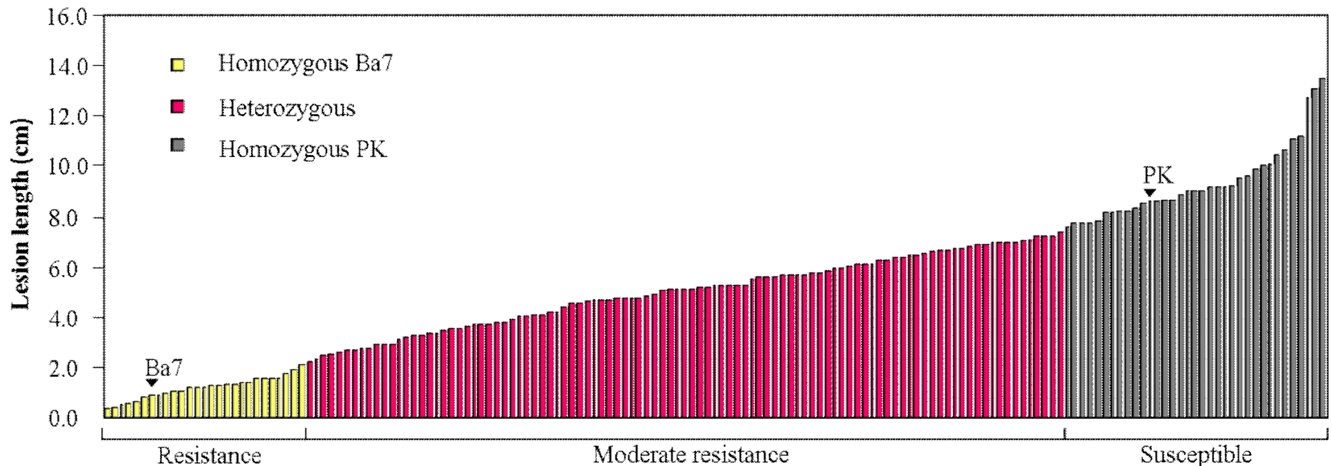

Figure 5. Distribution of LL of $BC_2F_{2:3}$ population after inoculation with TXO16. The red, yellow and blue bars represented homozygous Ba7 plants, heterozygous plants and homozygous PK plants respectively. Based on RM20590 genotypes, the Ba7 homozygotes, the heterozygotes and the PK homozygotes showed resistant, moderately resistant and susceptible phenotypes respectively.

Discussion

The study of identification, tagging, cloning and functioning of a gene can be performed effectively and rapidly in recent years. The near isogenic lines analysis [30] and bulked segregant analysis (BSA) [31] are used to rapidly identify DNA markers linked to the resistance genes. The use of these methods requires the construction of extremely resistant and susceptible bulks, and the reliability of the experimental results depends on the accuracy of phenotype validation [32]. In our study, we modified the BSA method by analysing individual plants from extremely resistant and susceptible groups. The advantage of this modified method is the higher resolution that it provides with consequent high tendency to the location of genes in comparison to the conventional BSA. In addition, we can monitor the linkage tendency of the molecular markers for the resistance gene, while traditional BSA does not allow us to do so because individuals are bulked for analysis. Although, after tagging the gene, we did not analyse the association between phenotypes and genotypes, nevertheless we could use the flanking molecular markers from the tagged gene for MAS directly.

The resistance gene which we identified in rice 'Ba7' was located on the long arm of chromosome 6. In this region, two other dominant genes (*Xa7* and *Xa27*) have been reported [15, 33]. *Xa7* was originally identified in rice cultivar 'DV85' [33]. A tightly-linked marker, RG1091, was mapped to the position 107.5 cM on the Rice Genome Research Program (RGP) map [34]. Other studies with various molecular markers including AFLP, SSR and STS indicated that *Xa7* was located at 107.3 cM [35-36]. Later, Chen et al. [37] reported the high-resolution mapping and the genetic prediction of resistance gene *Xa7*. This gene was mapped to the 0.21 cM interval between the STMS (GDSSR02) and the SSR marker (RM20593). The SSR markers RM20589, RM20590 and RM20591 were reportedly located between these two flanking markers. In our experiment, RM20590 was

identified as the closest *xa33*(t)-linked marker. Although, in this study, *Xa7* and *xa33*(t) shared common linked markers, they had shown different gene actions.

Based on our present study, *Xa7* and *xa33*(t) are not growth-stage dependent genes. They confer resistance to many Thai *Xoo* isolates at both seedling and booting stages. Sidhu et al. [33] reported that *Xa7* confers BB resistance only at the flowering stage. This is not the case with the Thai isolates. Seedling resistance is reportedly controlled by a number of known major genes conferring a high level of resistance throughout crop growth [38]. Adult plant resistance is characterised by a high level of resistance at the adult stage but the plants are very susceptible at their seedling stage [39]. Resistance at the seedling stage is more stable than resistance in adult plants [40]. Although *Xa7* and *xa33*(t) conferred BB resistance at seedling and tillering stages in our experiments, their resistance patterns against Thai BB isolates were different. The race specificity of the resistance genes indicated that *Xa7* and *xa33*(t) are not the same gene.

Amongst BB resistance genes identified on chromosome 6 including *Xa7*, *Xa27* and *xa32*(t), *Xa27* was reportedly located between RFLP markers RG424-RG162 (70.4-104.6 cM, Cornell map) on the long arm of chromosome 6, which is about 22.1 cM away from *Xa7*. It was originally found in wild rice *O. minuta* Acc 101141 [14] and introgressed into cultivated varieties. The gene *xa32*(t) was identified in wild rice *O. barthii* [6] and it was mapped on the terminal region of chromosome 6 at a distance of 9.3 cM from RM588 (16.1 Mb). Thus, *xa33*(t) in 'Ba7' was certainly different from *Xa27* and *xa32*(t) genes.

The gene *xa33*(t) conferred recessive gene action with incomplete dominance of susceptible allele because its heterozygous plants exhibited moderate susceptibility to *Xoo* strain. There are few reports on the genetics of incomplete susceptibility of BB found in rice. The *Xa27* gene conferred semi-dominant resistance to *Xoo* isolates PXO99 and T7174 in CO39 genetic background but provided complete resistance at the tillering stage in IRBB27 background while the seedling stage was susceptible. The inheritance of *Xa27* as a semi-dominant resistance gene was also observed in the genetic backgrounds of five parental lines of the Chinese hybrid rice when the plants were heterozygous at the resistance locus [15]. In the same way, *Xa21* and *Xa7* showed incomplete dominance in the heterozygous background of rice hybrid Minghui 63 by infection with GX325 and KS-1-21. The homozygous alleles were more resistant than the heterozygous ones [41]. Moreover, the *Xa4* resistance gene conferred from rice cultivar Teqing acted as a dominant resistance gene against *Xoo* strains CR4 and CXO8. On the contrary, it acted as a recessive factor against *Xoo* strain CR6 [42]. These incidents demonstrated that the *Xoo* strain which is used to evaluate the population, genetic background and developmental stage plays important roles in determining gene action.

In this study, we have identified the new BB resistance gene designated as *xa33*(t) in rice cv. 'Ba7'. The closely linked markers found will be useful for improvement of BB resistance through MAS in rice breeding programs.

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this paper was conducted at Rice Gene Discovery Unit, Kasetsart University, Khampaeng Saen campus, Nakhon Pathom, Thailand.

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