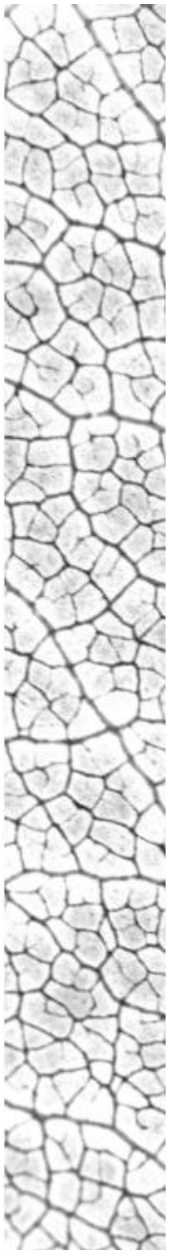

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Development of single nucleotide polymorphism markers for bacterial leaf blight resistance in rice

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Abstract
Bacterial leaf blight (BLB) disease, which is caused by Xanthomonas oryzae pv oryzae, is one of the devastating biotic stress in rice that results in yield losses. Cultivation of the resistant rice varieties has been proven as an environment-friendly and effective approach to address this problem. As such, this study developed a high quality and tightly linked marker of Xa7 gene, a resistant gene that controls BLB disease in rice. In this study, the mapping population of MR263 X IRBB7 was generated. The F2 population was used for genotyping purpose, while the F2:3 lines were employed for phenotyping purpose. Both genotyping and phenotyping data were used in bulk segregant analysis to narrow down the Xa7 region. A total 87 SNP markers were developed to genotype the mapping population. Out of 87 SNPs, only 65 SNPs exhibited acceptable call rates, and this was followed the Mendelian ration of F2 population (1:2:1). This study had successfully narrowed down the region of Xa7 from 118.5 kb to 58.5 kb, flanked by SNP_Xa7_14 and SNP_Xa7_31 that composed of 13 SNP markers. The developed SNP markers, which were tightly linked to Xa7 gene, emerge to be greatly significant in marker-assisted breeding activity to introgress BLB resistant gene into susceptible rice varieties. The application of the developed SNP marker is bound to enhance both efficiency and accuracy in the selection. The linkage drag phenomenon may be minimised as well as the marker is highly close to the target gene.

Keywords: bacterial leaf blight, SNP marker, *Xanthomonas oryzae pv oryzae*, Xa7

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INTRODUCTION

Bacterial leaf blight (BLB) has been reckoned as one of the most devastating diseases that affects the production of rice (Oryza sativa L.), which is caused by Xanthomonas oryzae pv. oryzae (Xoo). This horrendous blight is mainly noted in rice cultivation regions across Asia, northern Australia, western coast of Africa, and Latin America. The yield loss reported in Asia due to BLB ranged between 20 and 30% in moderate setting, and this figure could escalate up to 80% in favourable conditions (Kim 2018). Therefore, in order to hinder loss of yield, developing the resistant variant appears to be the most effective technique in controlling BLB that dismisses collateral data from farmers with nil adverse effect on the environment (Kim et al. 2015). At present, about 44 genes associated with resistance to BLB have been determined from a wide range of rice sources, namely artificially induced mutants, Oryza sativa, and wild rice (Busungu et al. 2016; Dilla-Ermita et al. 2017, Kim 2018). From these 44 genes, 27 are dominant genes, while the remaining 17 are recessive in their function.

Prior studies have clearly depicted that Xa7 refers to a dominant BB resistance gene, which was first found in rice cultivar DV85 (Sidhu et al. 1978). After that, this Xa7 gene had been transferred to IR24 and near-isogenic line (NIL) IRBB7 via backcross with IR24 that served as the recurrent parent (Ogawa et al. 1991). Previously, the gene was discovered between M1 and M3 in 2.7-cM interval (Porter et al. 2003). Chen et al. (2008) had narrowed down the Xa7 region to 118.5 kb interval that was flanked by RM20593 and GDSSR02 markers. Although the Xa7 gene has yet to be isolated, the avirulence (Avr) gene (AvrXa7) that correspondences to Xa7 gene has undergone cloning (Yang et al. 2000). This proves that the Xa7 gene is indeed a resistance

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gene that is durable due to a fitness penalty found in Xoo related to Xa7 adaptation (Vera Cruz et al. 2000). Hence, the introgression of Xa7 gene into susceptible rice varieties using effective and accurate molecular marker is required to develop BLB-resistant rice varieties.

In ensuring the success of a plant breeding program, it is vital to select the most viable material and discard harmful alleles. Marker assisted selection (MAS) is the application of genetic markers that aid in determining either harmful or desired alleles from a range of genotypes (Dubcovsky 2004). Upon being closely related to a desirable feature, the MAS are applied to choose the feature indirectly, thus minimises excessive labour, time, and money consumption. The MAS is beneficial in identifying multiple alleles within polygenic feature, costly or difficult features for phenotypical score, requirement of progeny evaluation because of lack of heritability and recessive nature, as well as being expressed late in the plant life (Koebner and Summers 2003). Nonetheless, indirect selection is bound to fail when recombination takes place between the marker and the desired feature/gene or if undesired alleles display linkage to the marker in germplasm accessions or if a marker has to be initially mapped in a new population (Varshney et al. 2005; Feng et al. 2019). Typically, recombination could occur at the least desired extent upon separating a genetic marker from its target gene exceeding 1–2 cM. Hence, there is a pressing needed to develop fine mapping to identify a tightly-linked marker to desired traits or to develop a functional marker. Tightly-linked markers are considered to be highly predictive of phenotype as they are very tightly linked to the target gene, hence the potential to reduce the occurrence of recombination between the favourable gene and the marker. As such, this study developed a single nucleotide polymorphism marker within the region of 118.5 kb positioned at the Xa7 gene.

MATERIALS AND METHODS

An F2 population composed of 267 plants that derived from the cross of IRBB7 X MR263 was employed for segregation analysis. The IRBB7 is NIL for the Xa7 gene, while the MR263 is a Malaysian susceptible commercial rice variety developed by Malaysian Agriculture Research and Development Institute (MARDI). The F2:3, which was generated and cultivated at MARDI Seberang Perai, had been used for phenotyping purpose.

Paired-end sequencing was carried out on Illumina® HiSeq platform to sequence the genomes of MR263 and IRBB7, with a read length of 150 bp at each end. The original sequencing data was acquired from high-throughput sequencing platforms recorded in image files, which were initially transformed to sequence the reads via base calling using the CASAVA software. The sequences and the corresponding sequencing quality information had been stored in a FASTQ file. Next, the effective sequencing data were aligned with reference sequence (Oryza sativa indica 93-11) with the BWA software only to retain "uniquely mapped reads". After that, both mapping rate and coverage were counted based on the alignment outcomes. The duplicates were discarded using SAMTOOLS (Li et al. 2009). The ANNOVAR refers to a commonly applied software program in variation annotation with multiple capabilities, including gene-based annotation, region-based annotation, filter-based annotation, and other functionalities. Thus, ANNOVAR was used in this study to annotate the identified SNPs (Wang et al. 2010). SNPs discovered within the 118.5 kb region of Xa7 were mined together with their 100 bp of their flanking region for the purpose of primer design.

The total genomic DNA of 267 F2 individuals were extracted using the protocol prescribed by Mace et al. The Agena MassARRAY platform (Agena Bioscience, San Diego, CA) was employed to genotype the DNA using the SNPs mined from IRBB7 and MR263 genomes within the Xa7 gene region. SNP assays were designed by using the MassARRAY Assay Design Software. Both extension and amplification reactions were performed using a minimum of 30 ng of DNA per sample and iPLEX Gold Reagent Kit by adhering to the protocol prescribed by its manufacturer. The SNP genotypes were scored using the MassARRAY Typer 4 analysis software.

The dominant pathotype of the causal pathogen Xoo had been cultured on peptone sucrose agar medium that comprised of 2% sucrose (w/v), 2.5% peptone (w/v), 0.05% K2PO4 (w/v), and 0.025% MgSO4·7H2O (w/v) at pH 7.0. The bacterial culture was suspended in sterile water and adjusted to a concentration of 1 x108 colony forming unit (CFU)/ml. Three replications of each F2:3 derived lines have been inoculated at maximum tillering stage by dipping the sterilized scissors in bacterial suspension and clipping off the leaves 2 - 3 cm from leaf tip (Kauffman et al. 1973). Each replication had 10 individuals. The disease severity was determined by measuring the lesion length on the infected leaves at 21 days after inoculation. The average length of the lesion was classified as resistant (R): < 5 cm, as resistant (MR): 5-10 cm, as moderate susceptible (MS): 10-15 cm and as susceptible (S): > 15 cm (Lore et al., 2011).

Based on the screening result, 30 F2:3 resistant lines (lesion length below 0.5 cm) and 30 F2:3 resistant lines (lesion length above 2.0 cm) were identified. The genotype data of their F2 derivative were compared between these two groups. The recombinant lines were determined based on the comparative results of the genotypic data.

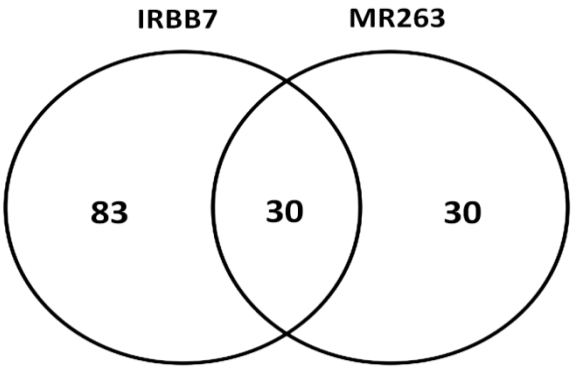

Fig. 1. Venn diagram of SNP distribution for IRBB7 and MR263

RESULTS

Sequence analysis of the 118.5 kb region of Xa7 revealed the existence of 113 and 60 SNPs from IRBB7 and MR263, respectively, upon being mapped to reference genome of Oryza sativa indica 93-11. A total of 83 and 30 SNPs were uniquely discovered in IRBB7 and MR263, respectively. The comparative target sequence analysis revealed that 30 SNPs were monomorphic. **Fig. 1** summarises the details of SNPs distribution. The SNP assay of those uniquely identified was designed, wherein only 87 SNPs were successfully designed based on their primer set and classified into three multiplex groups.

The genotyping analysis of 87 SNPs displayed that only 65 SNPs possessed acceptable call rates and followed the Mendelian ratio of F2 population (1:2:1) when assessed with Chi square analysis. The Chi square value of these 65 SNPs was lower than the tabulated value. Hence, only 65 SNPs out of the 87 genotyped across the 267 F2 individuals resulted in d.f = 2 with 0.05 confidence level (x2: 5.991), signifying that the SNPs adhered to 1:2:1 ratio of F2 population. The remaining SNPs with low call rates and deviated from the 1:2:1 ratio had been discarded from the bulk segregant analysis.

Based on the phenotyping analysis of F2:3 population, a group of resistant that consisted of 30 lines with lesion length lower than 0.5 cm, and a group of susceptible that composed of 30 lines with lesion length above 2.5 cm were generated (**Fig. 2**). Two recombinant lines were discovered in the resistant group, which succeeded in narrowing down the Xa7 region from those previously identified for 118.5-58.5 kb flanked by SNP_Xa7_14 and SNP_Xa7_31 (**Fig. 3**). **Table 1** presents a summary of the SNPs within 58.5 kb.

Fig. 2. Lesion length of the infection represents the resistant with the lesion length below 0.5 (left) and susceptible with the lesion length above 2.0 cm (right) toward Xoo inoculation

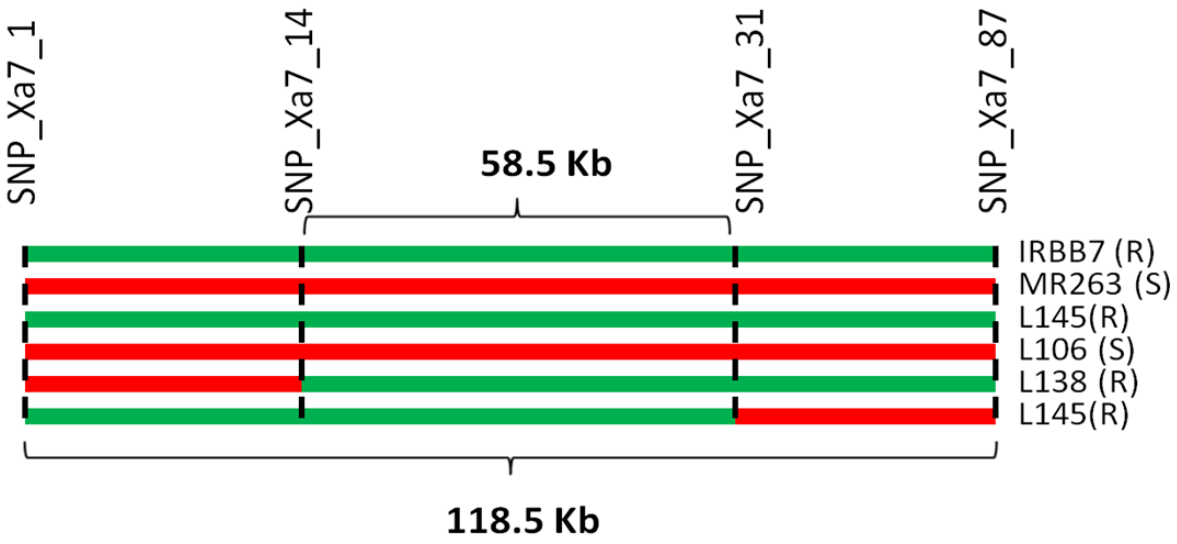

Fig. 3. Graphical genotype represents the region of 58.5 kb of Xa7 which involve in bacterial leaf blight resistant in rice

Table 1. Identified SNPs within 58.5 kb and their information

SNP ID	Position	Resistant allele	Susceptible allele	Annotated position
SNPXa7_14	29837816	A	G	Upstream
SNPXa7_15	29890341	G	A	Intergenic
SNPXa7_16	29890762	T	G	Intergenic
SNPXa7_17	29891311	T	C	Intergenic
SNPXa7_18	29891481	A	T	Intergenic
SNPXa7_19	29891924	C	T	Intergenic
SNPXa7_20	29892264	A	G	Intergenic
SNPXa7_21	29893172	T	A	upstream;downstream
SNPXa7_22	29893486	A	G	upstream;downstream
SNPXa7_27	29895584	A	G	Intergenic
SNPXa7_28	29895814	C	A	Intergenic
SNPXa7_30	29896203	A	C	Intergenic
SNPXa7_31	29896311	T	C	Intergenic

DISCUSSION

The Xa7 has been acknowledged as a broad spectrum and durable resistance gene. The outcomes based on the interaction of IRBB7 and almost all Xoo strains from Guangdong province, South China for 1991–2006 displayed that IRBB7 had good and stable resistance to Xoo pathogen (Chen et al. 2008). Rice lines that had Xa7 hindered BLB with presence of virulent Xoo strains, as reported in the Philippines between 1993 and 1995 (Vera Cruz et al. 2000). As a result of IRBB7 (Xa7) cultivation in field sites for a decade, it was found that the Xa7 gene continued to be highly effective, although the structure of the Xoo population had altered by increasing the amount of deleterious strains for IRBB7 and enhancing the aspect of aggressiveness towards rice with Xa7 absent from it (Leach et al. 2007). Based on the fine mapping performed in this study, some SNPs within the region Xa7 gene were developed to serve as an effective tool for the marker-assisted transfer of this resistant gene in programs linked with rice enhancement.

The implementation of tightly-linked marker for breeding programs may substantially enhance selection because of minimised false selection, as well as low recombination between gene and marker. The linkage drag is a concern in marker-assisted breeding. Due to far distance between random DNA markers and target genes, a larger donor segment may be introgressed into the recipient parent or backcross progeny upon application in MAB. When unfavourable genes are transferred together with the target gene, the performance of phenotype feature may be adversely affected (Cobb et al. 2019). Therefore, in the attempt of reducing the occurrence of linkage drag in marker-assisted breeding, Hospital (2001) prescribed the application of tightly-linked marker to introgressed gene within a huge population to retrieve double-recombinant genotypes. Feng et al. (2019) also shortened the region of pi21 in order to minimize the linkage drag phenomena, before being used in breeding programmed.

CONCLUSION

The developed SNP markers of Xa7 gene controlling BLB resistant gene are beneficial in marker-assisted breeding for introgression of resistant gene into susceptible rice variety. The application of the developed SNP markers can significantly increase

selection accuracy and reduce the linkage drag phenomenon in breeding program.

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