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# QTL-Seq Approach Identified *Pi63* Conferring Blast Resistance at the Seedling and Tillering Stages of Thai Indigenous Rice Variety "Phaladum"

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**Abstract:** Rice blast (BL) caused by *Magnaporthe oryzae* is a fungal disease causing significant yield losses in rice production worldwide. To overcome the breakdown of resistance by the rapid adaptation of pathogens, identifying resistance (*R*) genes or QTLs in indigenous rice, which harbors the *R* genes that co-evolved with the local pathogen race, is necessary. In this study, a recombinant inbred line (RIL) population derived from a cross between RD6 and Phaladum (PLD) was used to map quantitative trait loci (QTL) for BL resistance through a QTL-seq approach. A single QTL (*qBLchr4*) associated with BL resistance at the seedling and maximum tillering stages was mapped on the long arm of chromosome 4. Five genes, *LOC_Os04g0616600*, *LOC_Os04g0617900 (OsGLP4-1)*, *LOC_Os04g0619600 (OsRLCK161)*, *LOC_Os04g0620800 (Pi63)*, and *LOC_Os04g0621500*, were considered the candidate genes representing *qBLchr4*. Subsequently, the Kompetitive Allele-Specific PCR (KASP) markers specific for the SNP variant and position of each gene were designed for validation in the mapping population. These markers showed the high phenotypic variance explained (PVE) values in all testing methods and/or environments, signifying the major effect of *qBLchr4*. Among these markers, the *Pi63*-KASP marker explained the highest and most stable phenotypic variation across all testing methods and/or environments, with 84.18%, 80.34%, and 23.43% in the upland short row (USR) method, Sila environment, and Mueang environment, respectively. Therefore, *Pi63* was suggested to be the strongest candidate gene. These results represent the potential utility of future BL resistance breeding and/or pyramiding using marker-assisted selection (MAS).

**Keywords:** multiple-stage resistance; rice germplasm; rapid generation advance; upland short row; SNP index



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## 1. Introduction

Rice blast (BL) caused by *Magnaporthe oryzae* (*M. oryzae*) is a major and devastating disease in rice production throughout the world [1], in which outbreaks can result in decreasing rice grain yields by up to 10–35% [2]. For the sake of controlling this disease, the application of resistant varieties is regarded as the most effective and economical strategy [3]. However, cultivation of a resistant variety carrying a single resistance (*R*) gene for short periods often leads to resistance breakdown, as a consequence of pathogen adaptation [4]. To mitigate the breakdown of resistance, integrating multiple *R* genes (each with a different function) through a marker-assisted method is deployed for long-lasting and broad-spectrum resistance [5–8]. Therefore, there is an imperative need to explore new *R* genes to broaden the availability of a resistance source in rice breeding.

To date, approximately 100 BL *R* genes conferring resistance to *M. oryzae* have been identified [9]. The BL *R* genes are distributed across almost all rice chromosomes, except

chromosome 3 [10]. Most of the BL *R* genes reported have been identified in exotic rice cultivars and wild species of rice [10], which may not prove effective when exploited in rice breeding programs in Thailand, as a result of the race specificity of the *R* genes. On the other hand, the utilization of the *R* genes from Thai indigenous rice that co-evolved with local pathogen races [11] could offer an effective tool for rice breeding in Thailand. It is worth noting that among the identified *R* genes, several BL *R* genes have been discovered in indigenous rice varieties, such as *Pi-67* [12], *Pi54* [13], *Pigm* [14], *Pi-jnw1* [15], *Pi-d(t)1* [16], and *Pid(t)2* [16]. These *R* genes identified in indigenous rice have provided potential genetic resources in rice improvement for BL resistance. A good example of the utilization of such resistance resources is the Chinese indigenous rice Gumei 4 carrying the *Pigm* gene, which exhibited broad-spectrum resistance to *M. oryzae* and has been used as a BL resistance donor in rice improvement for the past five decades [14]. Although numerous *R* genes have been identified, many unexplored genes remain. Recently, a large number of indigenous rice varieties have been identified for disease resistance. Khannetah et al. [17] identified 4 and 34 Indian indigenous rice accessions as 'resistant' and 'moderately resistant' to bacterial blight (BB) disease, respectively. In Thailand, ten Thai indigenous lowland rice germplasms were also reported to confer resistance to BB, in which five of ten were identified as BB-resistant and showed some favorable agronomic traits [18]. Similarly, Chumpol et al. [19] identified ten Thai indigenous upland rice varieties as resistant to leaf blast, six of which were found to be resistant to both leaf blast and neck blast. Notably, in a previous study, we found that the Thai indica indigenous lowland rice variety Phaladum (PLD), collected from the Yasothon province in Northeastern Thailand, exhibited a high level of BL (both leaf blast and neck blast) and BB resistance, and possessed several superior agronomic traits. These results indicated that some resistant indigenous rice may contain the unidentified *R* gene. Thus, the identification of unexplored genes or QTLs conferring BL resistance in indigenous rice is necessary for their meaningful utilization in future resistant rice breeding programs.

For the rapid identification of genes or QTLs, a QTL-seq approach was developed by combining bulked-segregant analysis (BSA) and whole-genome resequencing of two DNA bulks of progeny showing contrasting phenotypes to identify genomic regions associated with focal traits, without marker developing and genotyping [20]. With these advantages, QTL-seq analysis successfully identified the genes or QTLs associated with several traits in rice [21–28], including blast disease [20]. In the present study, we employed the QTL-seq approach to identify the QTL for BL resistance in the recombinant inbred line ($F_6$ RIL) population derived from a cross between a BL-susceptible variety, RD6, and the BL-resistance indigenous variety PLD. The QTLs, as well as candidate genes for BL resistance, obtained in this study could provide a valuable tool for the molecular breeding of disease resistance in rice.

## 2. Materials and Methods

### *2.1. Development of Mapping Population*

Two parental varieties/accessions, RD6 and Phaladum (PLD), were used to generate the $F_6$ RIL population. RD6, the most favorable glutinous variety in Thailand, is both blast- and bacterial-blight-susceptible. PLD is a blast- and bacterial-blight-resistant variety that was obtained from the Rice Project, Khon Kaen University, Khon Kaen, Thailand. A single $F_1$ seed from an RD6/PLD cross was selfed to generate $F_2$ seeds; then, the mapping populations of 450 $F_6$ RILs were developed through the rapid generation advance (RGA) technique [29] (Figure 1).

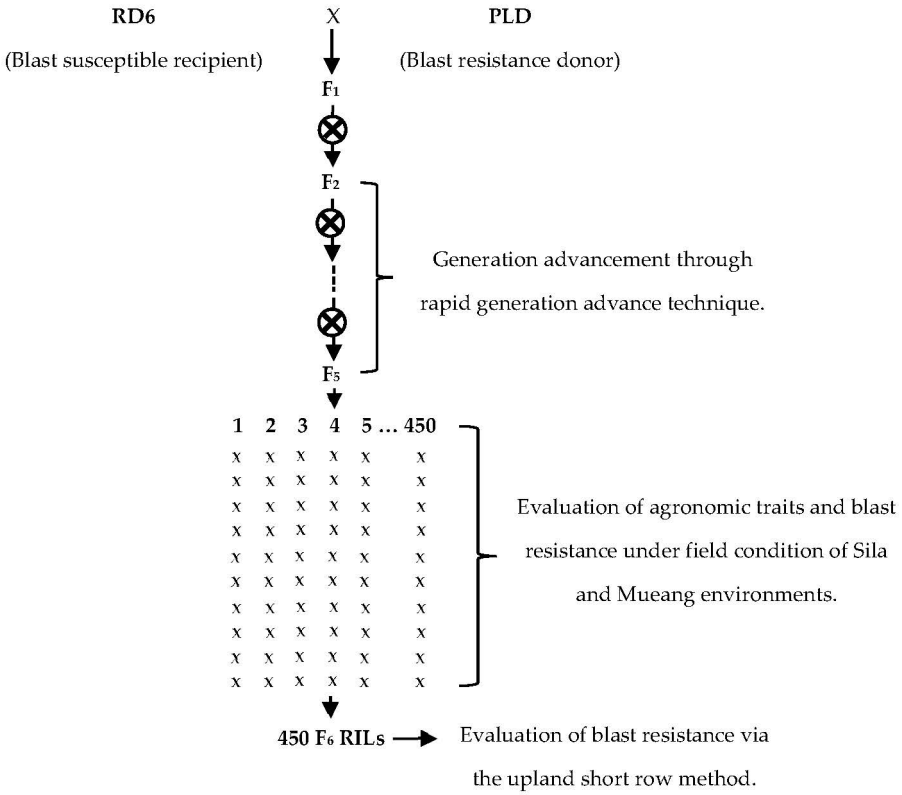


**Figure 1.** Scheme of the mapping population development from a cross between RD6 and PLD.

### *2.2. Evaluation of Blast Resistance*

To understand the genetic basis of BL in PLD, a total of 450 $F_6$ RILs derived from a cross between RD6 and PLD were investigated for the segregation of disease reaction against *M. oryzae*. Evaluations of BL resistance were conducted in both field conditions and via the upland short row (USR) method in 2020. For the field evaluations, thirty-day seedlings of each line were transplanted in single rows (2 m long), with a spacing of 25 cm between and within each row, in two environments, the Mueang and Sila sub-districts in Khon Kean, Thailand. Natural infection was utilized by transplanting the susceptible KDML105 variety in the experiment border rows. BL resistance was scored for ten plants at their maximum tillering stage (approximately 45 days after transplanting) using the 0–9 rating scale of the Standard Evaluation System for rice (SES) [30]. The testing lines with 0–5 scores were considered resistant (R) while 6–9 were deemed susceptible (S).

In addition to the field evaluation, BL resistance was also evaluated at the seedling stage of rice through the USR method, at the Agronomy Field Crop Station, Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand. The USR method evaluation was laid out in a randomized complete block design (RCB) with two replications. The local 'susceptible' KDML105 variety was planted in border strips 14 days before planting, to serve as a source of inoculum and as a spreader. Then, seeds of the parent, each RIL, and the 'resistant' check varieties (IR62266, P0489, and Jao Hom Nin) were sown in rows, 10 cm long and 7 cm apart. To ensure the uniform spread of the disease, the KDML105 variety was also planted after every ten test lines. Diseased plants of the BL-susceptible varieties (KDML105 and RD6), containing a mixture of natural *M. oryzae* strains, were collected from the Khon Kean rice fields. They were additionally used as an inoculum source, sprayed on all testing lines at the fourteen-day growth stage. Disease reactions were recorded ten days post-inoculation on a scale of 0–9 following the SES method [30], as described above.

### 2.3. Construction of Bulks, DNA Extraction, and Whole-Genome Resequencing

Contrasting phenotype bulks were generated for BL resistance based on disease reaction data of the RIL population. In constructing the contrasting phenotype bulks for BL resistance, 19 and 20 RILs showing the high and stable resistance and susceptible phenotypes were selected to generate resistant (BLR) and susceptible (BLS) bulks, respectively. The genomic DNA of each RIL in each bulk, as well as parental lines (PLD and RD6), were individually extracted using the GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Scientific™, Waltham, MA, USA). The quantity of DNA was measured and adjusted to equal concentrations. Each DNA bulk (BLR and BLS) was formed by pooling equal quantities of genomic DNA for each RIL. Later, the genomic DNA of two parents and two bulks was used to prepare DNA-seq libraries and was sequenced the whole genome via the Illumina HiSeq. 2500 platform (Illumina, Inc., Hayward, CA, USA).

### 2.4. QTL-Seq Analysis

The QTL-seq pipeline, developed by Takagi et al. [20], was used for QTL-seq analysis. First, to obtain high-quality reads, raw reads were trimmed by removing low-quality and adapter-contained reads. According to the requirement of reference genome generation for read mapping of two bulk samples, the high-quality reads of RD6 were aligned to the public reference genome of Nipponbare (IRGSP1.0) using a BWA aligner [31]. Subsequently, the RD6 reference genome was generated by substituting the base in the Nipponbare reference genome with the variants representing the RD6 parent. The high-quality reads of the two bulks were then aligned onto the RD6 reference genome to call DNA variants (SNP, single nucleotide polymorphism and Indel, small insertion/deletion) in BLR and BLS bulks. The SNP index calculation of both bulks was performed for each SNP position, as previously described [20,32]. SNPs with an SNP index <0.3 in both bulks were excluded, and the remaining SNPs (SNP index $\geq$0.3 in either bulk) were considered as the real SNPs, and were used for Δ(SNP index) calculation. The Δ(SNP index) was calculated by subtracting the SNP index of the BLR bulk from the SNP index of the BLS bulk. An average SNP index and Δ(SNP index) in a given genomic region were obtained from a sliding window analysis (with 2 Mb window size and 10 kb increment) to generate the plots of the distribution of average SNP index and Δ(SNP index) compared between the two bulks. Circos was applied for visualization of the plots of average SNP index and Δ(SNP index) compared between the two bulks [33]. The QTL for BL resistance was determined as a peak or valley of the SNP index plots that exhibited an average Δ(SNP index) higher than the statistical confidence intervals under the null hypothesis of no QTL ($p$-value < 0.05) [20].

### 2.5. Candidate Gene Annotation

Candidate genes presented in the detected QTL region were identified from the whole-genome resequencing data of the two parents (RD6 and PLD) that were aligned to the reference genome of Nipponbare using a BWA aligner. The alignment files obtained in the alignment step were converted to BAM files to identify the SNPs using Samtools [34]. The variance effect predictor (VEP: https://plants.ensembl.org/oryza_sativa/Tools/VEP) (accessed on 22 September 2021) was applied to determine the effect of the SNPs in each gene. Only the genes containing nonsynonymous SNPs were selected as candidate genes [25].

### 2.6. Development of KASP Markers and Marker-Trait Association Analysis

To validate the association of markers with the BL resistance phenotype, we designed five Kompetitive Allele Specific PCR (KASP) markers for *LOC_Os04g0616600*, *LOC_Os04g0617900* (*OsGLP4-1*), *LOC_Os04g0619600* (*OsRLCK161*), *LOC_Os04g0620800* (*Pi63*), and *LOC_Os04g0621500* to genotype 200 RILs (randomly selected from the 450 $F_6$ RIL mapping population). Each KASP marker was developed based on the specific SNP variant and position of each gene, i.e., a nonsynonymous SNP (C/G) at position 31,309,180 for *LOC_Os04g0616600*, a nonsynonymous SNP (G/A) at position 31,395,022 for *OsGLP4-1*, a nonsynonymous SNP (A/G) at position 31,478,782 for *OsRLCK161*, a nonsynonymous

SNP (C/A) at position 31,552,051 for *Pi63*, and a nonsynonymous SNP (T/C) at position 31,581,570 for *LOC_Os04g0621500*. The genotypic data from the KASP markers and the phenotypic data of 200 RILs were used for single-marker analysis [25].

## 3. Results

### *3.1. Evaluation of Blast Resistance and Plant Selection for Bulk Preparation*

For the rapid identification of QTLs capable of controlling blast (BL) resistance through the QTL-seq approach, we constructed a 450 $F_6$ RIL mapping population derived from a cross between the susceptible rice variety RD6 and resistance rice Phaladum (PLD). An $F_6$ RIL mapping population and parents were evaluated via the upland short row (USR) method (seedling stage) and in two environments (Sila and Mueang) during the maximum tillering stage for disease reaction against *M. oryzae*. Variations in resistance were observed, in which those in the Mueang population were less severe (Figure 2). Thus, we exclusively considered the disease reaction data obtained from USR and the Sila environment in this study. The results determined that the donor parent PLD was found to be resistant, with an average score of 3 and 2.57 for USR and Sila, respectively, while RD6 was found to be susceptible with an average score of 9 and 7.38 for USR and Sila, respectively. In the RIL population, the score ranged from 0.75 to 9 for USR and 1 to 8.78 for Sila. Across the two environments and/or methods, 203 RILs exhibited stable resistance against *M. oryzae*. The frequency distribution of $F_6$ RILs in USR and Sila appeared to follow a bimodal distribution (Figure 2). The segregation ratio of the $F_6$ RIL population tested under USR and Sila fitted to 1:1 ($X^2 = 1.91$, $p > 0.01$ and $X^2 = 2.72$, $p > 0.01$), with 244 resistant and 201 susceptible, and 250 resistant and 201 susceptible, respectively. These results suggested a single resistance gene conferring *M. oryzae* resistance in PLD. Additionally, correlations of the scores between USR and Sila were significant and strong ($r = 0.76$, $p < 0.01$) (Table S1), indicating that the single blast resistance gene of PLD contributes to both seedling and tillering stage resistance.

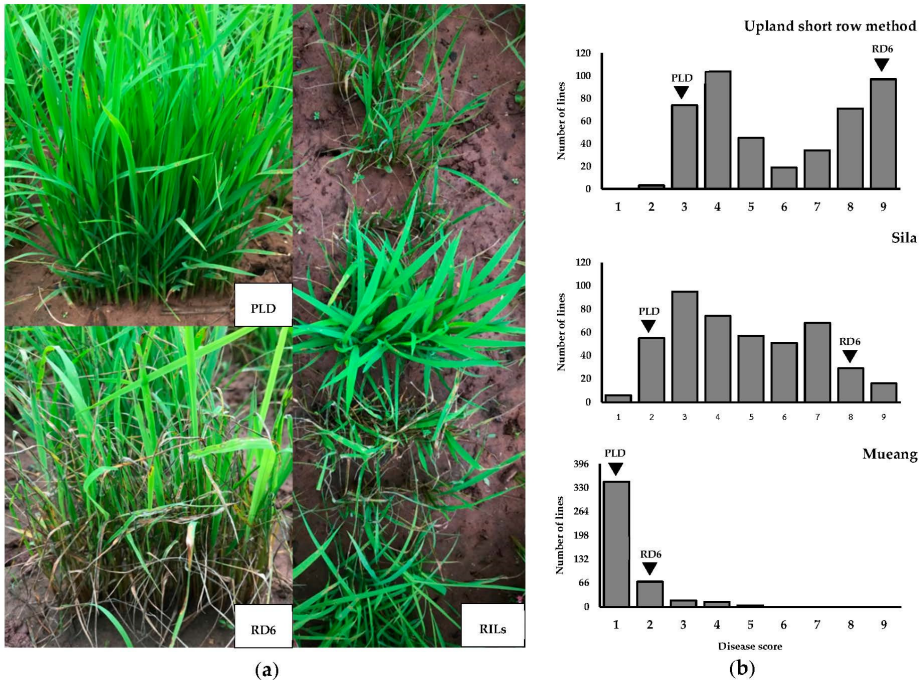


**Figure 2.** Blast (BL) phenotype in two parents and RILs: (**a**) the BL symptoms of the resistance parent PLD and susceptible parent RD6 and in RILs; (**b**) frequency distribution of disease reaction to a mixture of natural *M. oryzae* strains in 450 RD6/PLD $F_6$ RIL populations evaluated via USR method (top) and in two environments, Sila (middle) and Mueang (bottom). Disease scores of the parental lines (PLD and RD6) are indicated by a black triangle.

For QTL-seq analysis, we selected RILs that exhibited a consistent reaction (resistance or susceptible) to BL according to disease reaction data obtained from USR and Sila, but not Mueang, as disease infection was not severe. Disease scores from 19 resistance RILs and 20 susceptible RILs ranged from 1 to 3 and 7.13 to 9 for resistant (BLR) bulk and susceptible (BLS) bulk, respectively (Table S2).

### *3.2. QTL-Seq Analysis Identified QTL for Blast Resistance in PLD*

Four DNA libraries, consisting of two contrasting bulks (i.e., BLR-bulk (blast resistance) and BLS-bulk (blast susceptible)) and two parents (i.e., resistance donor PLD and susceptible recipient RD6) were sequenced with the whole genome using Illumina HiSeq. 2500. In total, approximately 109 million reads for BLR-bulk, 113 million reads for BLS-bulk, 106 million reads for PLD, and 110 million reads for RD6 (with 150 bp in length) were generated, which were equivalent to 16.30, 17.00, 15.90, and 16.40 Gb for BLR-bulk, BLS-bulk, PLD, and RD6, respectively. The average sequencing depths of BLR-bulk, BLS-bulk, PLD, and RD6 were 33.20, 33.00, 35.10, and 34.80, respectively. The alignment of the reads from two bulks and parents to the reference genome of Nipponbare revealed 76.12%, 73.02%, 86.65%, and 85.52% of read alignments in BLR-bulk, BLS-bulk, PLD, and RD6, respectively, corresponding to 94.53%, 94.43%, 92.49%, and 92.83% of rice genome coverage (Table 1). The high-quality reads of RD6 were used to generate the reference sequence of RD6. Then, read mapping against the reference sequence of RD6 was performed to identify the common SNPs between BLR-bulk and BLS-bulk for QTL-seq analysis. As a result, a total of 1,457,210 SNPs with read support of at least three reads were obtained in both bulks (Table 2). According to a read support criterion of at least 29 reads, 719,720 SNPs existing in both bulks were selected for further SNP index and Δ(SNP index) calculation (Table 2). The SNP index in each bulk and the Δ(SNP index) were physically plotted across all rice chromosomes to identify the QTL responsible for BL resistance in PLD (Figure 3).

**Table 1.** Summary of Illumina sequencing data of parental lines and resistant and susceptible bulks.

| Sample | Clean Reads | Clean Data (Gb) | Read Alignment (%) | Genome Coverage (%) | Average Depth |
|---|---|---|---|---|---|
| BLR-Bulk | 108,940,496 | 16.30 | 76.12 | 94.53 | 33.20 |
| BLS-Bulk | 113,062,190 | 17.00 | 73.02 | 94.43 | 33.00 |
| PLD | 106,155,076 | 15.90 | 86.65 | 92.49 | 35.10 |
| RD6 | 109,607,418 | 16.40 | 85.52 | 92.83 | 34.80 |

**Table 2.** Chromosome-wise distribution of single-nucleotide polymorphisms (SNPs) between two bulks.

| Chromosome | Length | Number of SNPs [1] | Selected SNPs [2] |
|---|---|---|---|
| 1 | 43,270,923 | 174,528 | 88,590 |
| 2 | 35,937,250 | 154,536 | 78,724 |
| 3 | 36,413,819 | 144,210 | 75,668 |
| 4 | 35,502,694 | 116,622 | 59,979 |
| 5 | 29,958,434 | 108,057 | 56,814 |
| 6 | 31,248,787 | 121,894 | 59,695 |
| 7 | 29,697,621 | 110,016 | 50,525 |
| 8 | 28,443,022 | 119,664 | 61,922 |
| 9 | 23,012,720 | 90,455 | 42,175 |
| 10 | 23,207,287 | 95,062 | 43,039 |
| 11 | 29,021,106 | 118,196 | 52,202 |
| 12 | 27,531,856 | 103,970 | 50,387 |
| Total | 373,245,519 | 1,457,210 | 719,720 |

[1] Number of SNPs with read support of at least three reads. [2] Selected SNPs with read support of at least 29 reads.

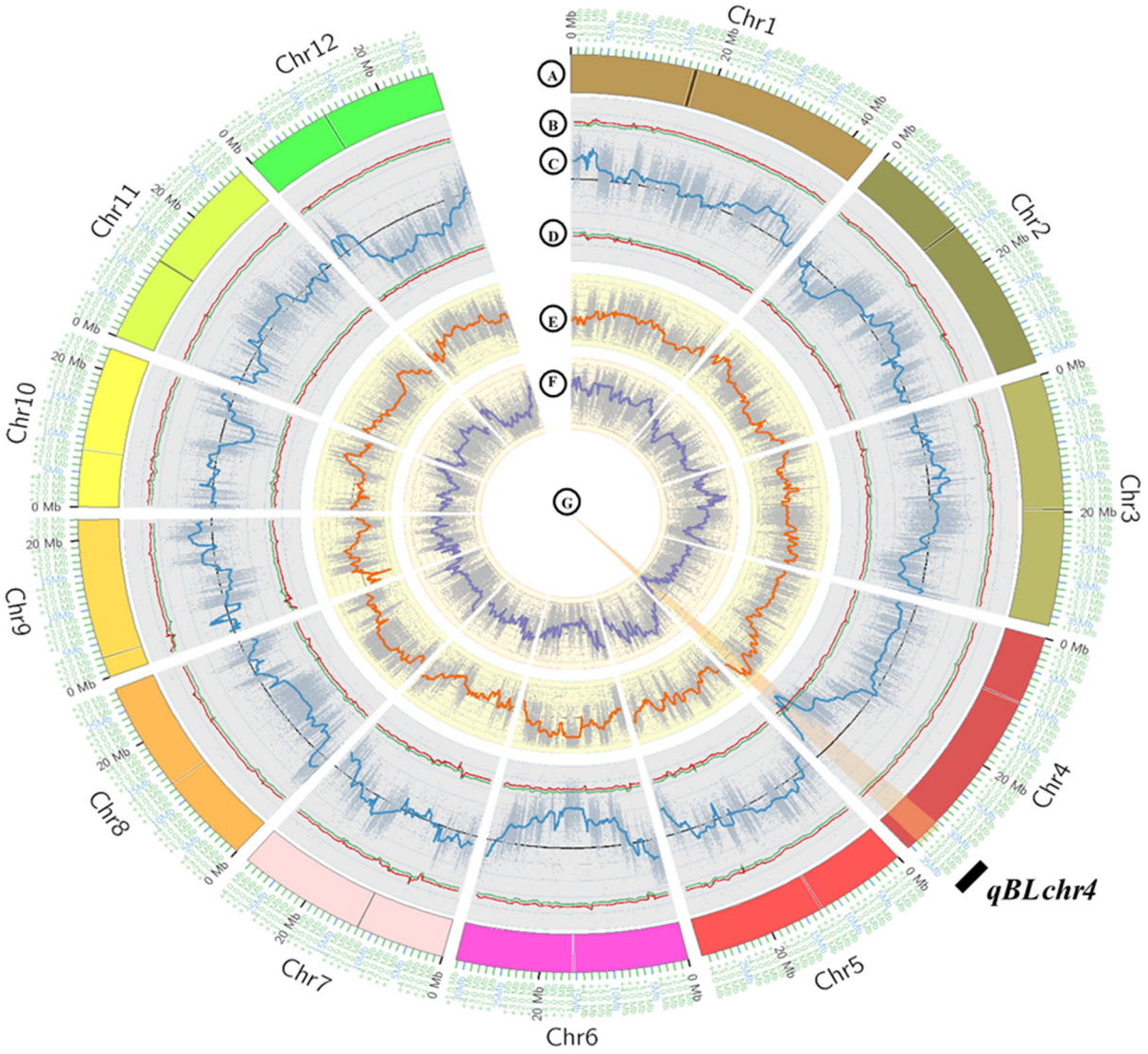


**Figure 3.** SNP index plots of BLR-bulk and BLS-bulk and plots of Δ(SNP index) obtained by subtracting between them: (A) pseudomolecules of Nipponbare reference genome (IRGSP 1.0); (B) upper probability values at 95% ($p < 0.05$; green line) and 99% confidence ($p < 0.01$; red lines); (C) plots of Δ(SNP index) with a window size of 2 Mb and 10 kb increment; (D) lower probability values at 99% ($p < 0.01$; red lines) and 95% confidence ($p < 0.05$; green line); (E) SNP index plots of BLR-bulk with a window size of 2 Mb and 10 kb increment; (F) SNP index plots of BLS bulk with a window size of 2 Mb and 10 kb increment; and (G) candidate genomic regions encompassing *qBLchr4* for blast resistance.

For the identification of putative QTLs, one QTL on the long arm of chromosome 4, namely *qBLchr4*, was detected based on the Δ(SNP index) plot that was greater than the threshold (confidence intervals > 95%) (Figure 3, Table 3). In the *qBLchr4* region, the average SNP index of BLR-bulk and BLS-bulk was 0.88 and 0.08, respectively, and the Δ(SNP index) was −0.80 (Figure 4, Table 3), indicating that plants in the BLR-bulk primarily contained the PLD-type genome in this QTL, whereas those in the BLS-bulk mostly contained the RD6-type genome.

**Table 3.** QTL detected for blast resistance in PLD.

| QTL | Chromosome | Location | | Interval (Mb) | Delta (SNP Index) | | Confidence Interval | |
|---|---|---|---|---|---|---|---|---|
| | | Start | End | | Min | Max | 95% | 99% |
| *qBLchr4* | 4 | 30,300,000 | 33,900,000 | 3.6 | −0.60 | −0.96 | −0.73 | −0.76 |

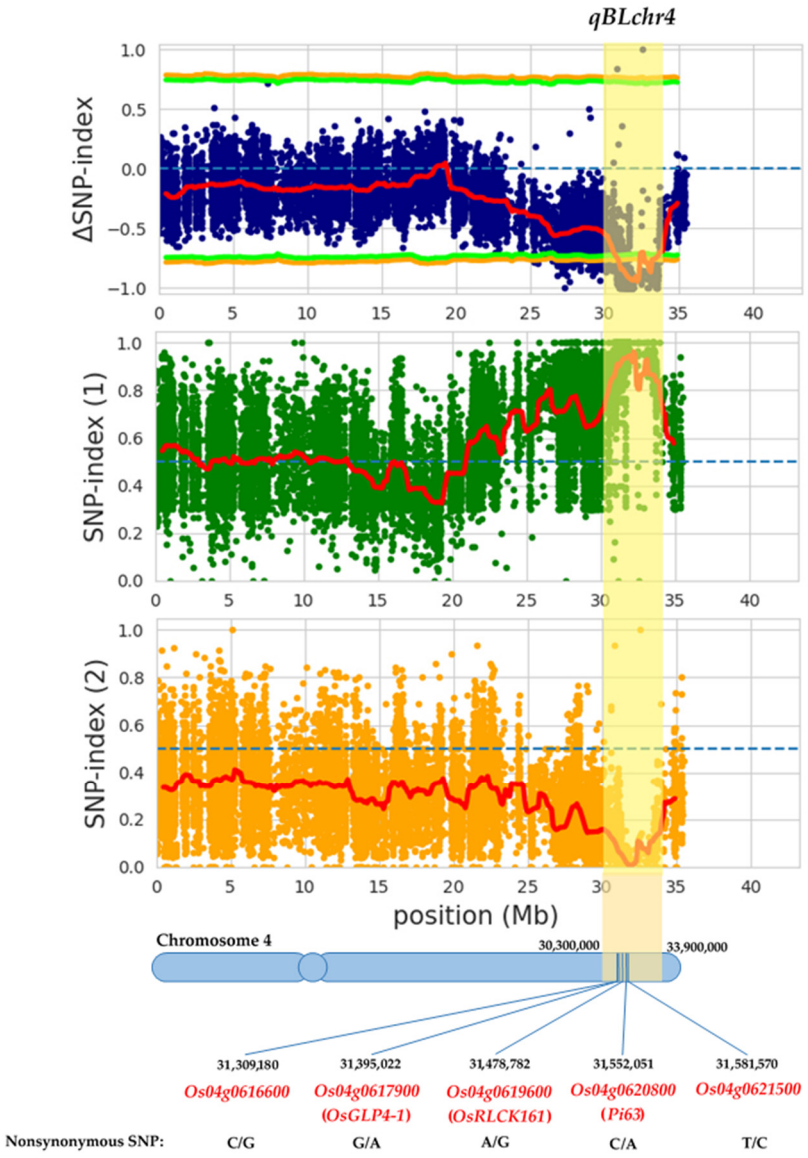


**Figure 4.** SNP index plots between resistance bulks (middle, BLR) and susceptible bulks (bottom, BLS) and Δ(SNP index) plots (top) on chromosome 4. The cream shade highlights the detected QTL regions with contrasting SNP indexes in two bulks.

*qBLchr4* was mapped to the 3.6 Mb region (between 30.3 and 33.9 Mb) with 339 annotated genes. Of these, 117 genes were in the 1 Mb region covering the valley of *qBLchr4* and only 41 genes contained nonsynonymous SNPs. Among them, five genes—LOC_Os04g0616600, encoding a serine/threonine protein kinase-related domain-containing protein; LOC_Os04g0617900 (*OsGLP4-1*), encoding a similar to germin-like protein subfamily 1 member 11; LOC_Os04g0619600 (*OsRLCK161*), encoding a similar to resistance protein candidate (Fragment); LOC_Os04g0620800 (*Pi63*), encoding a similar to H0714H04.10 protein; and LOC_Os04g0621500, encoding a disease resistance protein domain-containing protein—were known to be involved in disease resistance and were selected as the putative

candidate genes representing *qBLchr4* in this study (Figure 4). Notably, in this region, *Pi63* was previously reported as the BL *R* gene.

### 3.3. Validation and Confirmation of Identified QTL on Chromosome 4

Based on the *qBLchr4* region identified by QTL-seq analysis, five genes, *LOC_Os04g0616600*, *LOC_Os04g0617900* (*OsGLP4-1*), *LOC_Os04g0619600* (*OsRLCK161*), *LOC_Os04g0620800* (*Pi63*), and *LOC_Os04g0621500*, were selected as the putative candidate genes for BL (Figure 4). The KASP marker specific for each gene was then developed and used for genotyping in 200 RILs that were randomly selected from the 450 mapping population. The association between the KASP marker and BL resistance phenotypes was validated through single-marker analysis. As a result, the high phenotypic variance explained (PVE) value for all five markers was found in all testing methods and/or environments. These markers explained 58.58–84.18%, 55.81–80.34%, and 18.63–23.73% of phenotypic variation in USR, Sila, and Mueang, respectively (Table 4). It is noted that the PVE value was still high in Mueang, where the disease infection was not severe. Among the five markers, the marker specific to *LOC_Os04g0620800* (*Pi63*) explained the highest and most stable phenotypic variation across all testing methods and/or environments (Table 4).

**Table 4.** Single marker analysis of the five markers, *LOC_Os04g0616600*, *LOC_Os04g0617900* (*OsGLP4-1*), *LOC_Os04g0619600* (*OsRLCK161*), *LOC_Os04g0620800* (*Pi63*), and *LOC_ Os04g0621500*, and BL resistance of the $F_6$ RIL population.

| Markers | Chr | Upland Short Row Method | | | | Sila | | | | Mueang | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | LOD | PVE (%) | Add | Dom | LOD | PVE (%) | Add | Dom | LOD | PVE (%) | Add | Dom |
| LOC_Os04g0616600 | 4 | 36.86 | 58.58 | −1.93 | −0.69 | 34.14 | 55.81 | −1.74 | −0.46 | 9.64 | 20.63 | −0.44 | −0.35 |
| LOC_Os04g0617900 | 4 | 51.78 | 70.94 | −2.12 | −0.61 | 45.81 | 66.52 | −1.90 | −0.56 | 8.59 | 18.63 | −0.43 | 0.00 |
| LOC_Os04g0619600 | 4 | 73.28 | 82.48 | −2.22 | −1.65 | 65.76 | 79.10 | −2.01 | −1.36 | 11.30 | 23.73 | −0.46 | −0.49 |
| LOC_Os04g0620800 | 4 | 77.65 | 84.18 | −2.23 | −1.72 | 68.35 | 80.34 | −2.03 | −1.37 | 11.14 | 23.43 | −0.46 | −0.34 |
| LOC_Os04g0621500 | 4 | 75.06 | 83.20 | −2.25 | −1.21 | 66.23 | 79.33 | −2.03 | −0.94 | 9.66 | 20.68 | −0.44 | −0.11 |

Chr = chromosome, LOD = logarithm of odds, PVE = phenotypic variation explained, Add = additive effect, and Dom = dominant effect.

## 4. Discussion

Rice blast (BL) epidemics are a major challenge for rice production worldwide. According to the rapid adaptation of the pathogen to overcome the *R* genes of the host plant, the use of a resistance variety carrying a single *R* gene with broad-spectrum resistance or multiple *R* genes is considered to be the most effective and economical way to control this disease. To meet the demand, identification of the genes or QTLs responsible for BL resistance is crucial for rice breeding. Indigenous rice is considered to be a valuable source for BL resistance as it has harbored the *R* genes that co-evolved along with local pathogen races for a very long time [11]. Therefore, the main focus of the current study was to utilize the QTL-seq approach to locate QTLs for BL resistance in Thai indica indigenous rice Phaladum (PLD).

To identify the QTL for BL resistance, an $F_6$ RIL population derived from the two parents (RD6 and PLD), differing in BL resistance, was used for phenotyping in the QTL analysis. According to our correlation analysis of seedling stage resistance and tillering stage resistance for BL, a strong positive correlation of BL resistance between the seedling stage and tillering stage was found, implying that the BL *R* gene in PLD likely functions at both stages of growth. These many stages of resistance correspond with the previous result of Gerema et al. [35], which reported that two rice cultivars, Chewaga and Edget, conferred leaf blast resistance at both seedling and adult (maximum tillering) stages. Moreover, in addition to leaf and neck blast, panicle blast is also a common symptom caused by *M. oryzae* at the adult stage of rice. Three landraces, Yangmaogu, Jingnanwan, and Gumei 4, carrying the *R* genes *Pi64*, *Pi-jnw1*, and *Pigm*, respectively, were reported to confer both leaf blast (seedling) and neck blast resistance [15,36,37]. Similarly, Chumpol et al. [19] also reported that ten Thai indigenous upland rice varieties (including PLD) exhibited resistance to both

leaf blast and neck blast. In addition to the seedling and neck blast resistance of PLD, we additionally demonstrated the resistance of PLD at the tillering stage. The resistance expressed at many stages in growth is more desirable agronomically than that expressed at one particular stage (developmental stage-specific manner) [38]. Nevertheless, there are exceptions where plants are resistant at particular stages of growth. For example, a panicle blast resistance gene *Pb1* from indica rice variety Modan is susceptible to leaf blast during its young vegetative stage, but the resistance level then increases steadily and persists after heading [39,40].

In genetic analysis using 450 RILs, the mapping population showed a 1:1 segregation ratio. Generally, this 1:1 segregation ratio in the RIL population suggested the monogenic inheritance controlling the trait [41]. This follows previous reports that stated that the inheritance of BL resistance is controlled by a single gene [41–44]. The monogenic inheritance of BL resistance in PLD was confirmed by QTL-seq analysis. The results of this RIL population identified only one significant QTL (*qBLchr4*) on the long arm of chromosome 4. Furthermore, as high phenotypic variation explained (PVE) was achieved through single-marker analysis, *qBLchr4* was considered a major QTL in this study. This provides additional support for Miyamoto et al. [45], who identified major QTLs *qBFR4-1* (designated as *Pikahei-1(t)* and *Pi63* after fine mapping and cloning, respectively) for BL resistance, which explained 61.6% of phenotypic variance. Although numerous genes and QTLs for BL resistance have been identified [9,46], these sources have not been utilized effectively in rice breeding. This is because the genetic control of quantitative resistance is complex and the causal genes within resistance QTLs are still unknown [47]. Additionally, QTLs are frequently identified in the regions covering large DNA fragments, which might exhibit linkage drag, causing undesired traits when QTLs with different genetic backgrounds were pyramided into an improved commercial cultivar [47]. Identification of candidate genes underlying resistance QTLs will facilitate the effective utilization of these QTLs in rice breeding through marker-assisted selection (MAS) [47]. Among the molecular markers used in MAS, single-nucleotide polymorphisms (SNPs) have been considered to be the most promising, due to their high abundance in genomes and their potential for high-throughput automated genotyping [48].

In the present study, we identified five genes, *LOC_Os04g0616600*, *LOC_Os04g0617900* (*OsGLP4-1*), *LOC_Os04g0619600* (*OsRLCK161*), *LOC_Os04g0620800* (*Pi63*) and *LOC_Os04g0621500*, as the candidate genes representing *qBLchr4*. The KASP markers specific to the SNP variant and position of each gene were developed and used for validating the associations of markers with BL resistance phenotypes. According to the results of our single-marker analyses, the high phenotypic variance explained (PVE) value was found across each testing method and/or environment using all five markers, suggesting that these five markers can be potentially used in MAS for BL-resistant rice breeding. Previously, Yang et al. [48] also developed three KASP markers, each specific to SNP on the BL *R* gene: *Pita*; *Pik*; and *Pi2*. They were effectively used for BL resistance. It should be noted that the QTL identified in this study was stable across all testing methods and/or environments, even in the Mueang environment, where disease infection was minimal. Across the testing methods and/or environments, the *Pi63*-KASP marker explained the highest and most stable PVE value compared to other KASP markers used in this study. Therefore, *Pi63* is suggested to be the best candidate gene associated with BL resistance in PLD.

*Pi63* was previously reported as a BL-resistant gene capable of encoding a typical coiled-coil (CC) nucleotide-binding site and leucine-rich repeat (CC-NBS-LRR) protein [49], one of two NBS–LRR protein sub-classes [40]. The CC domain was found to have a function in pathogen recognition and defense signaling through intramolecular interactions [50]. The central NBS domain participates in nucleotide binding and plays an important role in transducing pathogen perception by LRR to resistance protein activation [40,51–53]. Additionally, Xu et al. [49] reported that *Pi63* exhibited partial resistance and isolate specificity to *M. oryzae* (resistance to two of four isolates used). This is in agreement with previous reports that many rice cultivars conferred partial resistance and isolate specificity

to *M. oryzae* [36,41,54,55]. According to the isolate specificity of *Pi63*, resistance can be overcome by the emergence of a new virulent isolate of *M. oryzae*. Thus, pyramiding *Pi63* from PLD with other *R* genes into a high-yielding rice variety, locally, through marker-assisted selection (MAS), is needed for broad-spectrum and durable resistance rice breeding in Thailand.

PLD is an indica indigenous lowland rice variety that confers resistance to local *M. oryzae* isolates and possesses some favorable agronomic traits, which provides beneficial breeding material for enhancing BL resistance and yield, as it might reduce genetic linkage dragging. To utilize this elite resistance source in future BL resistance rice improvement programs, selecting the RILs (obtained from a cross between RD6 and PLD that exhibited different BL resistance) showing different BL resistance with satisfactory agronomic traits may be attained. In the study herein, seven RILs conferred resistance to BL and possessed some desirable agronomic traits, i.e., early maturity (DTF, days to flowering), high tillering ability (TN, tiller number), short plant height (PH), and long and slender grains (SL, seed length and SW, seed width) (Table 5). These RILs may potentially be used as breeding materials for further BL resistance rice improvement programs in Thailand. Nevertheless, some agronomic traits of these RILs, such as the number of filled grains per panicle (NFGP), which will directly affect the grain yield per plant (GYP), indicated the need for improvement. To achieve this, marker-assisted backcrossing (MABC) is considered a promising approach to achieve both the desired agronomic traits with BL resistance.

**Table 5.** Blast scores and agronomic traits of selected RILs.

| Lines | Blast Score | | DTF | TN | PH | SL | SW | PL | NFGP | 1000 GW | GYP (g) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | USR | Sila | | | (cm) | (mm) | (mm) | | | (g) | |
| 100 | 3.50 | 1.67 | 107 | 11 | 136 | 10.30 | 3.12 | 26.33 | 119 | 28.73 | 36.12 |
| 124 | 3.00 | 1.11 | 71 | 14 | 118 | 9.99 | 2.68 | 27.00 | 131 | 21.23 | 29.35 |
| 126-1 | 4.00 | 2.67 | 91 | 14 | 159 | 10.10 | 2.66 | 29.43 | 144 | 23.16 | 22.85 |
| 132 | 2.50 | 1.00 | 71 | 12 | 166 | 10.32 | 2.66 | 30.50 | 185 | 25.12 | 29.62 |
| 238 | 4.00 | 1.00 | 70 | 17 | 143 | 9.73 | 2.64 | 26.75 | 183 | 23.47 | 30.44 |
| 335 | 4.00 | 1.56 | 89 | 13 | 126 | 9.97 | 2.74 | 26.50 | 141 | 24.51 | 32.00 |
| 361 | 3.00 | 2.00 | 90 | 10 | 150 | 10.39 | 2.80 | 26.67 | 143 | 26.80 | 29.49 |
| PLD | 3.00 | 2.57 | 69 | 13 | 126 | 9.54 | 2.91 | 26.35 | 145 | 26.35 | 28.16 |
| RD6 | 9.00 | 5.72 | 102 | 11 | 175 | 10.17 | 2.78 | 27.38 | 196 | 25.14 | 32.00 |

USR = upland short row method, DTF = days to flowering, TN = tiller number, SL = seed length, SW = seed width, PL = panicle length, NFGP = number of filled grain per panicle, GW = 1000-grain weight, GYP = grain yield per plant.

## 5. Conclusions

By applying QTL-seq analysis in the $F_6$ RIL population (RD6 × PLD), we successfully identified one major QTL (*qBLchr4*) on the long arm of chromosome 4, associated with BL resistance in both the seedling and tillering stages. Five genes—*LOC_Os04g0616600*; *LOC_Os04g0617900* (*OsGLP4-1*); *LOC_Os04g0619600* (*OsRLCK161*); *LOC_Os04g0620800* (*Pi63*); and *LOC_Os04g0621500*—were considered as candidate genes. The KASP marker designed for these genes was found to be effective in selecting plants with BL resistance. With the highest and most stable PVE value, *Pi63* was suggested as the best candidate gene for BL resistance. Moreover, the RILs selected from this population exhibited BL resistance and possessed desired agronomic traits; thus, they could provide good breeding materials for further BL-resistant rice breeding.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12081166/s1, Table S1: Correlation coefficient of blast score of the mapping population evaluated at seedling (USR method) and maximum tillering (Sila and Mueang environments) stages; Table S2: Blast score of RILs in the resistance bulk (BLR) and susceptible bulk (BLS) investigated via upland short row (USR) method and in the Sila environment.

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