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QTL-seq Identifies Genomic Regions Associated with Resistance to Dirty Panicle Disease in Rice

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Abstract: Dirty panicle disease is one of the most important diseases that can cause yield losses in rice production. Despite the severity of the disease, the molecular basis of resistance to the pathogen is poorly understood. Using QTL-seq with an F_2 population, we identified three genomic regions on chromosomes 1, 9, and 10, namely *qDP1*, *qDP9*, and *qDP10*. These regions are significantly associated with resistance to dirty panicle disease caused by two fungal pathogens, *Bioplaris oryzae* and *Cirvularia lunata*. *qDP1* was significantly associated only with resistance to *B. oryzae*, whereas *qDP9* and *qDP10* were significantly associated with both *B. oryzae* and *C. lunata*. We also developed KASP markers for each QTL detected and validated them in the F_2 population. The markers were able to explain phenotypic variation in a range of 5.87–15.20%. Twelve potential candidate genes with annotated functions as resistance-related genes were proposed. These candidate genes include those encoding RLK, MATE, WAK, NBS-LRR, subtilisin-like protease, and ankyrin repeat proteins. The results of this study provide insights into the genetic mechanism of dirty panicles in rice and will be useful for future breeding programs for dirty panicle resistance. This is the first report of QTLs associated with resistance to dirty panicle disease in rice.

Keywords: *Oryza sativa* L.; dirty panicle; *Cirvularia lunata*; *Bipolaris oryzae*; QTL-seq

Citation: Riangwong, K.; Aesomnuk, W.; Sonsom, Y.; Siangliw, M.; Unartngam, J.; Toojinda, T.; Wanchana, S.; Arikit, S. QTL-seq Identifies Genomic Regions Associated with Resistance to Dirty Panicle Disease in Rice. *Agronomy* **2023**, *13*, 1905. https://doi.org/10.3390/agronomy13071905

Academic Editor: HongWei Cai

Received: 2 July 2023
Revised: 15 July 2023
Accepted: 17 July 2023
Published: 19 July 2023

1. Introduction

Global climate change is expected to have various impacts on agricultural production worldwide [1]. Climate change may alter pathogen development and affect host-pathogen interactions, which may seriously threaten crop production, including rice [2]. Plant diseases are critical biotic stresses that could threaten the stability of rice production worldwide [3]. Dirty panicle disease is currently one of the most serious rice diseases in many rice-growing countries because the pathogens can directly infect the panicle and seeds, resulting in yield losses and affecting seed germination and seedling growth [4,5]. Other studies also reported that the pathogens usually infect the rice plants before and after harvest and consequently cause a reduction in rice yield and seed quality, such as germination rate [6]. Dirty panicle disease was found to cause yield losses of up to 60% in susceptible rice varieties [4,7]. Therefore, an important focus is developing an effective disease control approach.

Dirty panicle disease is a complex disease caused by several phytopathogenic fungi, including *Curvularia lunata*, *Cercospora oryzae*, *Bipolaris oryzae*, *Fusarium semitectum*, *Trichoconis padwickii*, and *Sarocladium oryzae* [8,9]. Most pathogens are seed-borne, but some can spread by wind and are considered epidemic diseases [10]. Symptoms of the disease vary depending on the environment and pathogen, such as black discoloration, dark brown spots, light to dark brown punctate spots, and light brown discoloration in the seed coat, endosperm, and embryo of infected seeds [10]. Dirty panicle disease was reported in rice and other tree plants, like coconuts. These were associated with the fungal pathogens *Alternaria burnsii*, *Fusarium clavum,* and *Fusarium tricinctum*. Symptoms include small brown to dark brown spots and discoloration of male flowers [11].

Various approaches have been recommended to control rice dirty panicle disease, including the use of fungicides, biological control agents, and plant extracts [12–15]. Farmers often tend to grow high-quality rice varieties with higher production, but most are highly susceptible to the disease. Disease control by spraying with fungicidal chemicals is still the most common method used by farmers. However, the continuous use of chemicals leads to undesirable effects such as fungicide resistance in pathogen populations, residues, and side effects for users, consumers, and the environment [16]. Therefore, using resistant varieties is an important solution to limit yield losses when the disease occurs. The use of resistance genes to develop disease-resistant varieties is much more efficient and safer than pesticides or other chemical control methods [17]. The advantages of using resistance genes in resistance breeding programs include efficient reduction in pathogen growth, minimal damage to the host plant, elimination of pesticide use by farmers, and environmental friendliness [18]. Unfortunately, this approach has not yet been applied to the disease control of dirty panicles in rice. To achieve this, the molecular background of the resistant trait must first be explored. To our knowledge, only a few studies are still underway on the genetic basis of the resistant phenotype to dirty panicle disease in rice.

QTL identification is one of the most important steps in plant breeding, which aims to find markers closely linked to the QTL that control the desired traits. DNA markers closely associated with agronomically important genes can be used as molecular tools or marker-assisted selection (MAS) in plant breeding [19,20]. In addition, the current technique, called next-generation sequencing (NGS), provides convenient, rapid, and inexpensive whole-genome sequencing. This technique has discovered many differences in single nucleotide polymorphisms (SNPs) and insertion/deletion (In/Del) sequences. Currently, an approach called QTL-seq is widely used to rapidly and accurately determine a candidate genomic region for target genes or QTLs. This method involves combining high-throughput whole-genome resequencing and bulk segregant analysis (BSA) in a segregating population, e.g., recombinant inbred lines (RILs) or F_2 [21]. This method searches for genomic regions that differ in the single nucleotide polymorphism (SNP) index between two bulk samples with significantly different phenotypes [21]. QTL-seq has contributed to the rapid identification of genes or QTLs in various crops such as chickpea [22], barley [23], soybean [24], canola [25], cucumber [26,27], and sesame [28]. This method has also been used to identify genes or QTLs that control resistance to plant diseases in various crops, such as powdery mildew in cucumber [29], rust and late leaf spot in peanuts [30], late leaf spots in peanuts [31], and Phytophthora crown spot in squash [32]. In rice, QTL-seq was used to identify the QTL associated with several important phenotypes, e.g., grain length [33], dwarfism [34], cooked grain elongation [35], various responses to abiotic stresses [36–38], and resistance to biotic stresses such as bacterial leaf streak [38], blast [21,39], and brown planthopper [40]. In this study, we identified genomic regions and candidate genes associated with resistance to panicle disease caused by *Bipolaris oryzae* and *Curvularia lunata*. We have also developed molecular markers for screening resistance to dirty panicle disease in rice. The results of this study will be useful for breeding programs to develop resistant cultivars to dirty panicle disease.

2. Materials and Methods

2.1. Mapping Population and Plant Growth Conditions

A cross between two parents, IR68586-F2-CA-31 (DH103; highly resistant to dirty panicle disease) and improved-RD47 (susceptible to dirty panicle disease), was made to generate the F_1 population. A total of 600 F_2 lines derived from the F_1 plants were used as the mapping population to identify QTLs associated with dirty panicle (DP) disease resistance using the QTL-seq approach. Each F_2 plant was grown in a 10-inch pot filled with clay from a rice field. All plants were grown for 60 days, then the plantlets (tillers) of each F_2 line were divided into two groups and transplanted into three separate pots for each group. Each F_2 line (three pots per line) was used separately for screening with *Cirvularia lunata* and *Bipolaris oryzae* isolates. Of these, 462 lines were inoculated with *Bipolaris oryzae* (isolate NPT0508), and 412 lines were inoculated with *Cirvularia lunata* (isolate CMI01161). The experiments were conducted under greenhouse conditions at Kasetsart University, Kamphaeng Saen, Nakhon Pathom, Thailand, from May to September 2019.

2.2. Inoculum Preparation and Pathogen Inoculation

A virulent isolate of *B. oryzae* (NPT0508) and a virulent isolate of *C. lunata* (CMI01161) were selected to represent the diversity of pathogens [41,42]. The pathogens were provided by the Department of Plant Pathology, Faculty of Agriculture at Kamphaen Saen, Kasetsart University, Kamphaeng Saen, Nakhon Pathom, Thailand, courtesy of Associated Professor Dr. Jintana Unartngam. The inoculum was grown under constant fluorescent light at 28 °C for 14 days on a potato dextrose agar plate (Himedia, Mumbai, India) (Figure S1). A spore suspension was obtained from the cultures by adding 10 mL of sterilized water to each plate and gently scraping with a sterilized loop. The spore concentration was adjusted to 1×10^5 spores/mL using a hemocytometer before inoculation. The conidial suspensions of each isolate were inoculated by spraying five panicles per replication of each F_2 line on the first day after the panicles were fully exserted. The experimental design was a completely randomized design (CRD). For each F_2 line, three replicates were used for both *C. lunata* and *B. oryzae* inoculations.

2.3. Evaluation of Dirty Panicle Disease

Disease severity was assessed 21 days after inoculation. The panicle symptom was rated on five scales from 1 to 5, where 1 = 1–25% of <0.1 mm black spots on panicle (resistant), 2 = 25% of >0.1 mm black spots on panicle (moderately resistant), 3 = 50% large spots on panicle (moderately susceptible), 4 = 50–75% large spots on panicle (susceptible), and 5 $\geq$ 75% large spots on panicle (highly susceptible) (Figure S2).

2.4. Construction of Resistance and Susceptible Bulks and Whole Genome Sequencing

For each inoculation, 25 F_2 lines with the highest resistance and another 25 lines with the highest susceptibility to dirty panicle disease were obtained and divided into resistant and susceptible groups, respectively. Genomic DNA was isolated from the young leaves of each selected F_2 plant using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). An equal amount (50 ng/mL) of the high-quality genomic DNA of each F_2 individual in each group was pooled to form R-bulk and S-bulk, respectively. The pooled DNA from the two bulks, along with DNA from the two parents, was sent to the Novogene Bioinformatics Institute (Beijing, China) for whole-genome sequencing using the Illumina HiSeq 2500 platform (Illumina, Inc., San Diego, CA, USA).

2.5. QTL-seq Analysis

The QTL-seq pipeline version 2.2.2 (https://github.com/YuSugihara/QTL-seq; accessed on 1 July 2023) was used for QTL-seq analysis [21,43]. DH103 was used to generate the parental reference genome to map the reads of the two bulks. The reads of the R-bulk and S-bulk were aligned to the parental genome to identify DNA variants, including single nucleotide polymorphisms (SNPs) and insertions/deletions (indels). Only

SNPs at positions in both bulks were used to calculate the SNP index, as previously described [21,43,44]. The Δ(SNP index) was then calculated as the difference in the indices between the two bulks at each position. The confidence intervals of the Δ(SNP index) were determined using ten thousand permutations. The distribution of the SNP index in each bulk and the Δ(SNP index) estimated in the genomic interval using a sliding window of 2 Mb in steps of 10 kb were recorded for all rice chromosomes. The positions of SNPs corresponding to genomic regions associated with resistance to the fungal pathogens were determined from the sliding window plots, with a confidence cut-off of a *p*-value of 0.05.

2.6. Candidate Gene Annotation

Candidate genes were annotated for each significant QTL interval based on MSU Rice Genome Annotation Project Release 7 (http://rice.uga.edu; accessed on 1 July 2023), and candidate genes with nonsynonymous variations were determined. The effects of the obtained SNPs were verified using Variant Effect Predictor (VEP: https://plants.ensembl.org/Oryza_sativa/Tools/VEP; accessed on 1 July 2023).

2.7. KASP Marker Development and Validation

Kompetitive Allele-Specific (KASP) markers were developed based on the selected SNPs. KASP assays were performed using the LGC Genomics manual (http://www.lgcgenomics.com, accessed on 1 July 2023). The KASP reaction was performed in a 96-well format and set up as a 5 µL reaction with 2 µL of DNA template, 0.075 µL of assay mix, and 2.5 µL of master mix. Amplification was started at 94 °C for 5 min, followed by 10 cycles at 94 °C for 20 s and at 61 °C for 60 s (touchdown to 61 °C, decreasing by 0.6 °C per cycle), followed by 27 cycles at 94 °C for 20 s, 55 °C for 30 s, and a rest period at 37 °C for 1 min. After amplification, the fluorescence signals of the final PCR products were read using the QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA).

2.8. Statistical Analysis

Single-marker analysis was performed with linear regression in R (http://www.r-project.org, accessed on 1 July 2023) using the lm() function. Genotype data were the genotypes of KASP markers, and phenotype data were the DP scores of the F_2 population for inoculations with *C. lunata* and *B. oryzae.*

3. Results

3.1. Disease Evaluation of Parental Lines and F_2 Population and Bulk Construction

The severity of DP symptoms in parental lines DH103 and improved-RD47 differed significantly, as the symptoms of DP caused by both pathogens were less pronounced in DH103 than in improved-RD47 (Figure 1A–D). Disease scores evaluated among the F_2 lines showed a normal distribution for *C. lunata* and *B. oryzae* inoculations (Figure 1B,D). The DP disease scores of the F_2 plants inoculated with the *B. oryzae* isolate NPT0508 ranged from 1.13 to 4.42, with an average of 2.56. The average disease scores of DH103 and improved-RD47 were 1.15 and 4.30, respectively. Similarly, disease scores of F_2 plants inoculated with the *C. lunata* isolate CMI01161 ranged from 1.00 to 4.08 with an average of 2.53, and the average disease scores of DH103 and improved-RD47 were 1.33 and 4.73, respectively. Twenty-five highly resistant F_2 plants and 25 highly susceptible F2 plants were selected to produce a resistant bulk (R-bulk) and susceptible bulk (S-bulk), respectively, for both *C. lunata* and *B. oryzae* inoculations (Figure 1B,D).

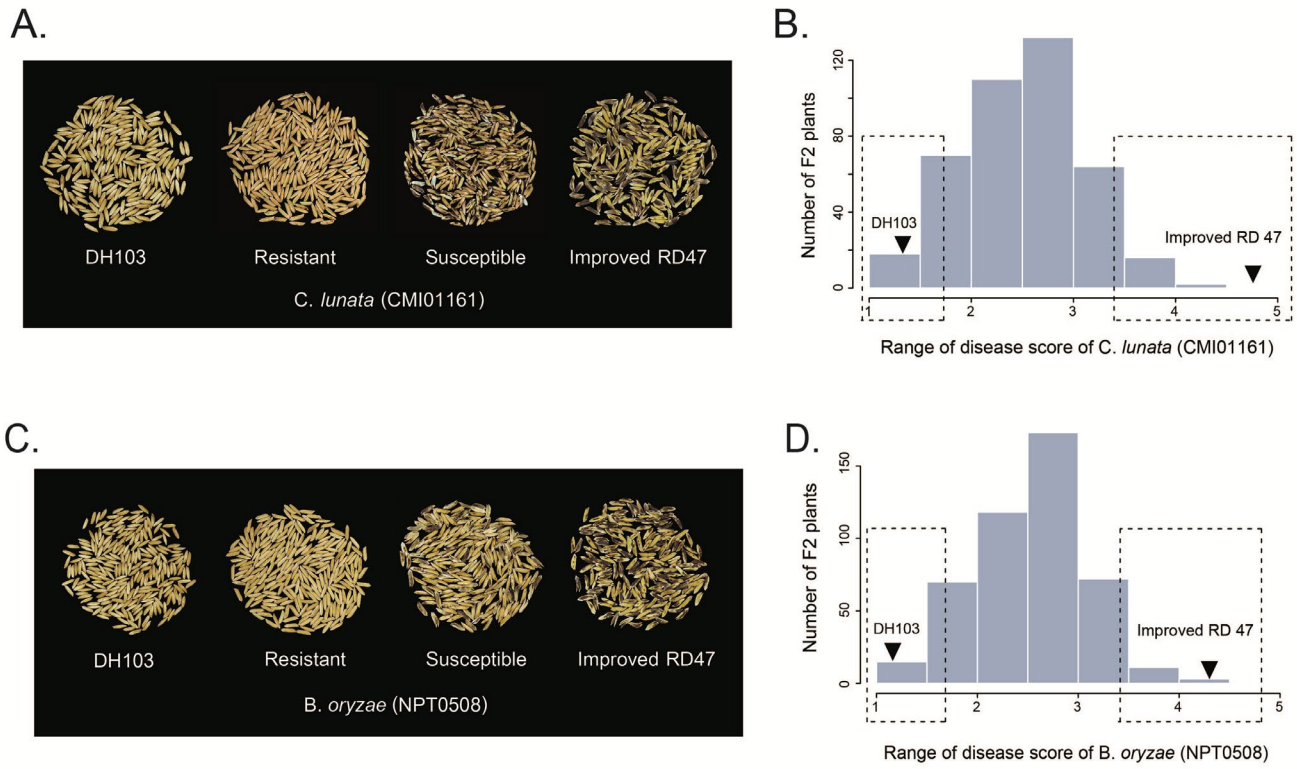

Figure 1. Dirty panicle disease phenotype in parental lines and F_2 population. (**A**,**C**), dirty panicle disease symptom in the resistant parent (DH103) and susceptible parent (improved-RD47), and the representative F_2 lines in the resistant bulk and susceptible bulk, inoculated with *B. oryzae* isolate NPT0508 and *C. lunata* isolate CMI01161. Frequency distribution of the resistance phenotype in parental lines and the F_2 population inoculated with (**B**) *C. lunata* and (**D**) *B. oryzae*. The dashed rectangle shows the cut-off for resistant and susceptible individuals for DNA bulking and sequencing.

3.2. Whole-Genome Sequencing of the Parental Lines and Bulks of F_2 Population

A total of 83.43 million reads and 80.61 million reads were generated with an average depth of approximately 24.18 and 24.28 for improved RD47 and DH103, respectively. Similarly, the sequencing of bulks resulted in 86.01 million reads for *B. oryzae*_R-bulk, 86.56 million reads for *B. oryzae*_S-bulk, 82.55 million reads for *C. lunata*_R-bulk, and 71.56 million reads for *C. lunata*_S-bulk (Table 1). The low-quality sequences were filtered and trimmed out to obtain high-quality sequences. Cleaned reads were obtained in each sample as 68.24 million reads in DH103, 68.41 million reads in improved-RD47, 72.28 million reads in *B. oryzae*_R-bulk, 73.31 million reads in *B. oryzae*_S-bulk, 68.50 million reads in *C. lunata*_R-bulk, and 56.93 million reads in *C. lunata*_S-bulk (Table 1). According to the alignment of clean reads of each sample onto the Nipponbare reference genome, a total of 10.22 Gb of clean data (24× coverage) was obtained for improved-RD47 and DH103, 10.82 Gb clean data (25× coverage) for *B. oryzae*_R-bulk, 10.98 Gb clean data (25× coverage) for *B. oryzae*_S-bulk, 10.25 Gb clean data (24× coverage) for *C. lunata*_R-bulk, and 8.50 Gb clean data (21× coverage) for *C. lunata*_S-bulk (Table 1).

The numbers of genome-wide SNP variants detected in the R-bulk and S-bulk for *B. oryzae* and in the R-bulk and S-bulk for *C. lunata* after mapping cleaned reads onto the DH103 genome reference were 1,469,859, 1,434,485, 1,296,083, and 1,304,282, respectively. The numbers of InDel variants detected in the R-bulk and S-bulk for *B. oryzae* and those in the R-bulk and S-bulk for *C. lunata* were 372,828, 367,684, 329,251, and 329,566, respectively. The SNP variants used in the QTL-seq analysis were the common SNPs identified in both the R-bulk and S-bulk of B. oryzae and in the R-bulk and S-bulk of *C. lunata*, with read support of at least eight reads (Table 2 and Figures S3 and S4).

Table 1. Summary of Illumina sequencing data of parental lines and F_2 bulks for *B. oryzae* and *C. lunata* inoculations.

Sample	Raw Reads (Million)	Cleaned Reads (Million)	Cleaned Base (Gb)	Alignment (%)	Genome Coverage (%)	Average Depth (×)
*B. oryzae*_R-bulk	86.01	72.28	10.82	96.85	98.18	25.06
*B. oryzae*_S-bulk	86.56	73.31	10.98	96.86	98.16	25.39
*C. lunata*_R-bulk	82.55	68.50	10.25	94.67	99.97	23.95
*C. lunata*_S-bulk	71.56	56.93	8.50	94.63	99.90	21.23
DH103	80.61	68.24	10.22	95.35	99.97	24.28
Improved-RD47	83.43	68.41	10.22	93.50	99.94	24.18

Table 2. Summary of the numbers of SNPs and Indels detected in each rice chromosome based on the read alignment on DH103 parent reference for *B. oryzae* and *C. lunata* inoculations.

Chr.	Length	Selected SNPs (Depth ≥ 8) (*B. oryzae*)	Selected Indels (Depth ≥ 8) (*B. oryzae*)	Selected SNPs (Depth ≥ 8) (*C. lunata*)	Selected Indels (Depth ≥ 8) (*C. lunata*)
1	43,270,923	42,284	13,949	45,528	13,593
2	35,937,250	20,343	6671	19,328	5940
3	36,413,819	42,885	13,796	49,078	14,203
4	35,502,694	30,112	9227	36,558	10,077
5	29,958,434	25,226	7828	26,094	7615
6	31,248,787	18,825	5930	15,123	4460
7	29,697,621	9209	3145	8994	2871
8	28,443,022	28,998	8477	27,976	7651
9	23,012,720	17,329	5570	17,591	5203
10	23,207,287	19,337	5807	20,431	5802
11	29,021,106	21,131	6167	22,879	6129
12	27,531,856	15,963	4840	16,793	4821
Total	373,245,519	291,642	91,407	306,373	88,365

3.3. QTL-seq Analysis

QTL-seq analysis of dirty panicle disease (DP) resistance was performed for both *B. oryzae* (NPT0508) and *C. lunata* (CMI01161) inoculations. The SNP-index plots were generated for all 12 chromosomes for *B. oryzae* (NPT0508) and *C. lunata* (CMI01161) inoculations. To identify candidate genomic regions associated with resistance to DP caused by *C. lunata* and *B. oryzae*, Δ(SNP index) values were calculated across the genome using moving window averages of 2 Mb window intervals with a 10 kb increment. Significant genomic positions were identified with a statistical significance of $p < 0.05$. The QTL-seq analysis results for *B. oryzae* inoculation identified four candidate regions on chromosomes 1, 9, and 10, with Δ(SNP index) of 0.34–0.38, 0.26–0.27, and 0.26–0.33, respectively. The QTL on chromosome 1 peaked at 7.61 Mb, that on chromosome 9 peaked at 19.12 Mb, and that on chromosome 10 peaked at 20.36 Mb (Figure 2 and Table 3). The QTL-seq analysis results for the inoculation of *C. lunata* isolate CMI01161 identified two candidate regions on chromosome 9, peaking at 20.5 Mb with a delta SNP index of 0.26–0.31, and on chromosome 10, peaking at 21.4 Mb with a delta SNP index of 0.26–0.33. These two QTLs were found to overlap with those identified for *B. oryzae* inoculation. Therefore, we determined these two QTLs to be the common QTLs associated with DP resistance against both pathogens. We named the QTLs associated with resistance to DP on chromosomes 1 (at 4.45–8.74 Mb), 9 (at 17.14–20.94 Mb), and 10 (at 18.86–22.20 Mb) as *qDP1*, *qDP9*, and *qDP10*, respectively (Table 3).

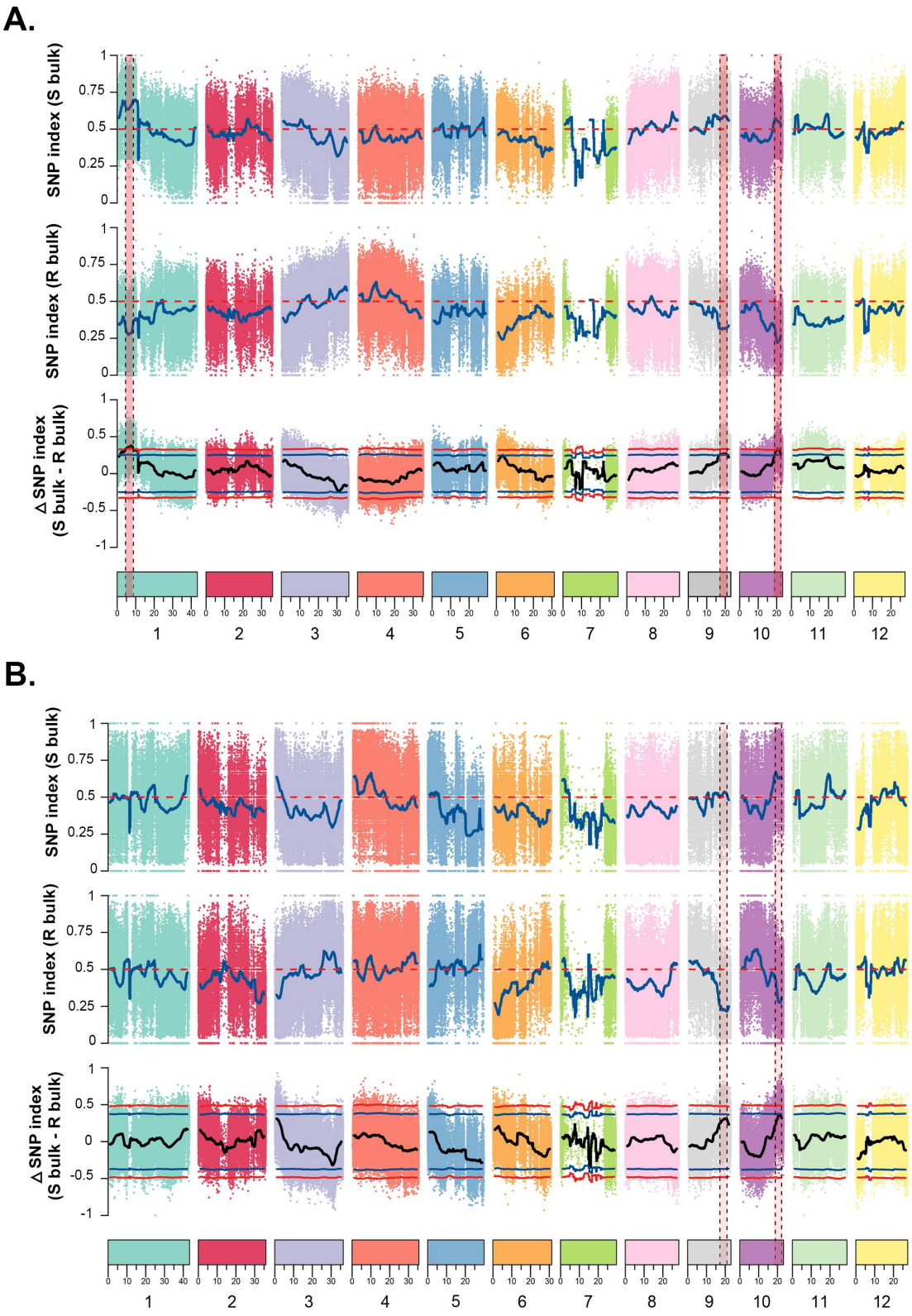

Figure 2. Plots of the SNP index of S-bulk and R-bulk and Δ(SNP index) across 12 rice chromosomes. (**A**) Plots for *B. oryzae* inoculation. (**B**) Plots for *C. lunata* inoculation. The pairs of blue and red lines in the Δ(SNP index) plots represent 95% and 99% confidence intervals, respectively. Genomic regions significantly associated with dirty panicle disease resistance are highlighted by orange shading and flanked by dashed vertical lines.

Table 3. Summary of the genomic region associated with resistance to dirty panicle disease caused by *B. oryzae* and *C. lunata*.

	B. oryzae					*C. lunata*				
QTL	**Chr.**	**QTL Region (Mb)**	**Δ(SNP Index)**	**Confidence Interval (95%)**	**Peak (Mb)**	**Chr.**	**QTL Region (Mb)**	**Δ(SNP Index)**	**Confidence Interval (95%)**	**Peak (Mb)**
qDP1	1	4.63–8.60	0.34–0.38	0.25	7.61					
qDP9	9	17.61–20.92	0.26–0.27	0.25	19.12	9	17.70–21.30	0.26–0.31	0.25	20.5
qDP10	10	18.86–22.20	0.26–0.33	0.25	20.36	10	19.28–22.20	0.26–0.33	0.25	21.4

3.4. Marker Development and Validation of the Detected QTLs

Based on the results of the QTL-seq analysis, we developed KASP markers to validate the identified QTL regions. Twenty-four KASP markers were designed based on the SNPs identified within *qDP1*, *qDP9*, and *qDP10*. We used these KASP markers to genotype the whole F_2 population and performed single marker analysis (SMA). Based on the SMA results, we considered the markers with phenotypic variance explained (PVE) values of $\geq$5% and *p*-values < 0.001. As a result, 12 markers with PVE values ranging from 5.87–15.20% were retained, including two markers on chromosome 1, six markers on chromosome 9, and four markers on chromosome 10 (Table 4 and Table S1). The marker that showed the highest association with resistance to *B. oryzae* was DP_10_20653028 on chromosome 10, with a phenotypic variance explained (PVE) value of 13.59%. The marker that showed the highest association with resistance to *C. lunata* was DP_9_20080072 on chromosome 9, with a PVE value of 15.20%.

Table 4. Single marker analysis of the markers for resistance to dirty panicle disease against *B. Oryzae* and *C. lunata* in the F_2 population with DH103 as a resistant contributing parent.

Marker	**Pathogen**	**Tested Sample**	**Chr.**	**Position of SNPs**	***p*-Value**	**PVE (%)**
DP_1_7200175	*B. oryzae*	213	1	7,200,175	1.69×10^{-7}	12.19
DP_1_7690710	*B. oryzae*	219	1	7,690,710	2.58×10^{-7}	11.54
DP_9_18493128	*B. oryzae*	224	9	18,493,128	6.94×10^{-7}	10.52
	C. lunata	197	9	18,493,128	6.65×10^{-5}	7.85
DP_9_19918713	*B. oryzae*	229	9	19,918,713	2.13×10^{-4}	5.87
	C. lunata	193	9	19,918,713	2.40×10^{-6}	11.02
DP_9_20080072	*B. oryzae*	233	9	20,080,072	1.56×10^{-5}	7.77
	C. lunata	200	9	20,080,072	1.14×10^{-8}	15.20
DP_9_20370866	*C. lunata*	198	9	20,370,866	1.15×10^{-7}	13.39
DP_9_20572426	*C. lunata*	190	9	20,572,426	7.28×10^{-7}	12.27
DP_9_20857648	*B. oryzae*	226	9	20,857,648	7.77×10^{-6}	8.56
	C. lunata	193	9	20,857,648	8.11×10^{-8}	14.02
DP_10_20377830	*B. oryzae*	214	10	20,377,830	1.36×10^{-6}	10.44
DP_10_20653028	*B. oryzae*	259	10	20,653,028	9.25×10^{-10}	13.59
	C. lunata	236	10	20,653,028	2.08×10^{-7}	10.90
DP_10_20837907	*B. oryzae*	215	10	20,837,907	2.9×10^{-6}	9.78
DP_10_21635840	*C. lunata*	200	10	21,635,840	2.86×10^{-4}	6.44

3.5. Putative Candidate Genes for Dirty Panicle Disease

We annotated genes within each detected QTL associated with resistance to *B. oryzae* and *C. lunata*. We considered the genes containing nonsynonymous SNPs with contrasting SNP indices comparing R bulk and S bulk. Twelve genes were identified, including three for *qDP1*, eight for *qDP9*, and one for *qDP10* (Table 5). These included LOC_Os01g12720 (protein kinase domain-containing protein), LOC_Os01g12950 (ubiquitin-conjugating enzyme) and LOC_Os01g13800 (receptor-like protein kinase 5) for qDP1, LOC_Os09g29284 (multi antimicrobial extrusion protein MatE family protein), LOC_Os09g29510 (OsWAK receptor-like protein kinase), LOC_Os09g30380 (TIR-NBS resistance protein), LOC_Os09g30458 (subtilisin-type protease), LOC_Os09g33800 (arabinogalactan protein), LOC_Os09g34150 (NBS-LRR disease resis-

tance protein, LOC_Os09g34160 (NBS-LRR disease resistance protein) and LOC_Os09g34280 (ankyrin repeat-containing protein) for *qDP9*, and LOC_Os10g38800 (serine/threonine protein kinase-related domain-containing protein) for *qDP10*.

Table 5. Candidate genes containing nonsynonymous SNPs in the identified genomic region for *B. oryzae* and *C. lunata* resistance.

QTL	Chr.	Gene Locus	Description	Position (bp)	Variant Effect	R-Bulk Variant Rate	S-Bulk Variant Rate	Pathogen Resistance
qDP1	1	LOC_ Os01g12720	Protein kinase domain-containing protein	7,041,499	TCA>TTA	0.31	0.74	*B. oryzae*
qDP1	1	LOC_Os01g12950	Ubiquitin-conjugating enzyme	7,208,599	CGC>CAC	0.27	0.79	*B. oryzae*
qDP1	1	LOC_Os01g13800	receptor-like protein kinase 5	7,740,829	AAC>AGC	0.39	0.76	*B. oryzae*
qDP9	9	LOC_Os09g29284	Multi antimicrobial extrusion protein MatE family protein	17,780,448	CTT>CAT	0.11	0.56	*B. oryzae*
				17,784,135	TTT>TCT	0.32	0.67	*C. lunata*
qDP9	9	LOC_ Os09g29510	OsWAK80–OsWAK receptor-like protein kinase	17,950,756	ACC>ATC	0.26	0.67	*B. oryzae*
						0.11	0.60	*C. lunata*
qDP9	9	LOC_Os09g30380	Similar to tir-nbs resistance protein	18,494,719	ATG>AAG	0.34	0.69	*B. oryzae*
				18,493,128	GCT>GTT	0.12	0.71	*C. lunata*
qDP9	9	LOC_Os09g30458	Similar to Subtilisin-type protease	18,559,879	AAT>AGT	0.23	0.70	*B. oryzae*
				18,559,449	GTG>GCG	0.19	0.65	*C. lunata*
qDP9	9	LOC_Os09g33800	Similar to arabinogalactan protein	19,956,506	CAT>CTT	0.24	0.59	*B. oryzae*
				19,957,424	CGG>CCG	0.34	0.72	*C. lunata*
qDP9	9	LOC_Os09g34150	NBS-LRR disease-resistance protein	20,159,557	CAT>CCT	0.45	0.74	*B. oryzae*
qDP9	9	LOC_Os09g34160	NBS-LRR disease-resistance protein	20,163,293	GGA>GAA	0.14	0.70	*C. lunata*
qDP9	9	LOC_Os09g34280	Ankyrin repeat-containing protein	20237630	CAG>CGG	0.15	0.56	*B. oryzae*
				20,239,736	AAC>AGC	0.07	0.65	*C. lunata*
qDP10	10	LOC_Os10g38800	Serine/threonine protein kinase-related domain-containing protein	20,653,028	ATG>ACG	0.28	0.73	*B. oryzae*
				20,654,458	CGC>CAC	0.35	0.67	*C. lunata*

4. Discussion

Modern agriculture has relied too much on the use of pesticides to control disease infections in crops. In rice, dirty panicle disease and other diseases caused by fungi are mainly controlled by the use of fungicides [45]. There are also reports of the use of biocontrol methods to control phytopathogenic fungi, such as antagonistic bacteria [46] and mycoviruses [45]. As an alternative, breeding a resistant variety has been proposed as a sustainable, environmentally friendly, and efficient method of disease control [47]. However, the genetic basis of resistance to dirty panicle disease in rice is currently unknown. Thus, it has been a burdensome effort for molecular breeding development in disease management systems. Although several pathogens can cause dirty panicle disease in rice, we focused on two fungal pathogens, *Bipolaris oryzae* and *Cirvularia lunata*. These two fungi were given particular attention because they were found to be the major pathogens of rice dirty panicle disease, especially in Thailand [12].

QTL-seq has been effectively used to identify regions controlling many traits of interest in rice and other crops [48]. In this study, we used the QTL-seq approach to identify genomic regions associated with resistance to dirty panicle (DP) disease in rice that was caused by *B. oryzae* and *C. lunata*. The segregating population used in this study was an F_2 population derived from a cross between DH103 and improved-RD47. The parent DH103 is a rice variety derived from a double haploid (DH) population from a cross between an indica variety, IR62266, and a japonica variety, CT9993 [49]. DH103 has been used as a donor for several abiotic stresses, e.g., drought tolerance and salt tolerance [50]. However, the use of this rice variety for dirty panicle disease resistance has not been reported. In this study, DH103 highly resisted the dirty panicle disease caused by both pathogens. Another parent, improved-RD47, is a bacterial leaf blight resistance variety derived from RD47, which is a high-yield rice variety from Thailand. The improved RD47 variety is susceptible to dirty panicle disease. The size of each bulk of F_2 plants in this study (n = 25 for each bulk) is sufficient to identify the major loci associated with resistance [21]. Meanwhile, genome coverage obtained in each bulk was 21–25 folds, within the range of 6–80 folds in previously reported studies [21,32,51]. Using QTL-seq, we successfully

identified three genomic regions on chromosomes 1, 9, and 10 associated with dirty panicle disease resistance. Since the QTLs on chromosomes 9 and 10 were found to be overlapped in both inoculations of the two pathogens, we suggest that these two QTLs could be used in rice breeding programs for dirty panicle disease resistance. Currently, there is no other report of QTLs/genes associated with DP disease resistance in rice. To our knowledge, this is the first report of QTLs associated with resistance to DP disease caused by *C. lunata* and *B. oryzae*.

The Kompetitive Allele-Specific PCR (KASP) marker is a genotyping platform that achieves high throughput in a time- and cost-efficient manner [52]. Validation of the detected QTLs using the KASP markers developed for each detected QTL indicated promising results. Two markers on chromosome 10 (DP_10_20377830 and DP_10_20837907) showed a highly significant association with the resistance to dirty panicles caused by *B. oryzae*. On the other hand, two markers on chromosome 9 (DP_9_20370866 and DP_9_20572426) and one marker on chromosome 10 (DP_10_21635840) were found to be significantly associated with dirty panicle disease caused by *C. lunata*. Four markers located on chromosome 9 (DP _9_18493128, DP _9_19918713, DP_9_20080072, and DP _9_20857648), and one marker (DP _10_20653028) located on chromosome 10 were found to be significantly associated with resistance to both fungal pathogens. The dirty panicle is a complex disease caused by various pathogens. Thus, markers that can detect the phenotype caused by multiple pathogens can be beneficial.

The annotation of potential candidate genes in the detected QTL regions has resulted in several putative resistance-related genes. The candidate genes on chromosome 1 included LOC_Os01g12720 (protein kinase domain-containing protein), LOC_Os01g12950 (ubiquitin-conjugating enzyme), and LOC_Os01g13800 (receptor-like protein kinase 5), which were considered putative candidate genes for resistance to the fungus *B. oryzae*. Eight candidate genes on chromosome 9, i.e., LOC_Os09g29284 (multi antimicrobial extrusion protein MatE family protein), LOC_Os09g29510 (OsWAK receptor-like protein kinase), LOC_Os09g30380 (TIR-NBS resistance protein), LOC_Os09g30458 (subtilisin-type protease), LOC_Os09g33800 (arabinogalactan protein), LOC_Os09g34150 (NBS-LRR disease resistance protein), LOC_Os09g34160 (NBS-LRR disease resistance protein), and LOC_Os09g34280 (ankyrin repeat-containing protein) were considered candidate resistance genes to both *B. oryzae* and *C. lunata*. Additionally, a candidate gene on chromosome 10, LOC_Os10g38800 (serine/threonine protein kinase-related domain-containing protein), was considered a candidate resistance gene to both *B. oryzae* and *C. lunata*. In addition, several functional SNPs (SNPs with a nonsynonymous effect) determined in these genes were proven to be significantly associated with dirty panicle disease resistance. Receptor-like kinases (RLKs) play a critical role in response to stimuli and serve as key components in plant disease resistance [53]. Wall-associated kinases (WAKs) are positive regulators of resistance to fungal diseases in several plant species [54]. Ubiquitin-conjugating enzyme is one of the three enzymes involved in the ubiquitin-26S proteasome system (UPS) [55]. In plants, ubiquitination plays a critical role in the response to biotic and abiotic stimuli [56]. MATE (Multidrug and Toxic Compound Extrusion or Multi-Antimicrobial Extrusion) transporters have been directly or indirectly implicated in the mechanisms of detoxification of heavy metal toxicity and disease resistance [57,58]. *NBS-LRR* genes encode NBS-LRR proteins that help plants defend themselves against pathogen invasion [59]. TIR-NBS has been shown to play a role in the basal defense response of plants [60]. Subtilisin-like proteases (or subtilases) have been reported to play a role in plant defense responses against a wide variety of pathogens [61]. Ankyrin repeat-containing proteins have been reported to play a role in both disease resistance and antioxidant metabolism [62]. Serine/threonine-protein kinases have also been reported to be involved in disease resistance in rice [63]. Most of the candidate genes identified in this study have promising functions in the context of disease resistance in plants. However, further studies, such as gene expression analysis, are likely needed to verify the function of these candidate genes.

5. Conclusions

In this study, we successfully used QTL-seq to identify three QTLs associated with resistance to dirty panicle disease in rice. Many resistance-related genes (R genes) were annotated within the discovered QTL regions. To the best of our knowledge, this is the first report of genetic information on resistance to dirty panicle disease in rice. The results of this study will be useful for further elucidation of the genetic control of dirty panicles in rice, and the markers developed in this study may be useful in rice breeding programs for dirty panicle resistance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13071905/s1, Figure S1: The fungal pathogens used in this study to screen for resistance to dirty panicle disease; Figure S2: Rating scores (1–5: resistance-susceptible) for evaluation of dirty panicle disease resistance; Figure S3: SNP density within 1-kb window size across 12 rice chromosomes detected in R-bulk and S-bulk of the *C. lunata* inoculation; Figure S4: SNP density within 1-kb window size across 12 rice chromosomes detected in R-bulk and S-bulk of the *B. oryzae* inoculation; Table S1: Details of KASP markers used in the study.

Author Contributions: Conceptualization, S.A., T.T. and S.W.; formal analysis, K.R., W.A. and Y.S.; writing—original draft preparation, K.R.; writing—review and editing, K.R. and S.W.; supervision, S.A., M.S., J.U., T.T. and S.W.; funding acquisition, S.W. and T.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Science and Technology Development Agency, Thailand (NSTDA grant numbers: P-17-50001 and P-18-51456) and the National Research Council of Thailand (NRCT) (NRCT-RTA/812/2563). K.R. was supported by a full scholarship from the Ministry of Higher Education, Science, Research, and Innovation, Thailand.

Data Availability Statement: The data generated are available in the article and Supplementary Materials.

Acknowledgments: The authors would like to thank the Innovative Plant Biotechnology and Precision Agriculture Research Team, BIOTEC, NSTDA, and the Rice Science Center, Kasetsart University, for rice material support. The authors would also like to thank the Department of Plant Pathology, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, for *Bipolaris oryzae* and *Cirvularia lunata* isolate support.

Conflicts of Interest: The authors declare no conflict of interest.

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