*Article*

# QTL Mapping of Resistance to Bacterial Wilt in Pepper Plants (*Capsicum annuum*) Using Genotyping-by-Sequencing (GBS)

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**Abstract:** Bacterial wilt (BW) disease, which is caused by *Ralstonia solanacearum*, is one globally prevalent plant disease leading to significant losses of crop production and yield with the involvement of a diverse variety of monocot and dicot host plants. In particular, the BW of the soil-borne disease seriously influences solanaceous crops, including peppers (sweet and chili peppers), paprika, tomatoes, potatoes, and eggplants. Recent studies have explored genetic regions that are associated with BW resistance for pepper crops. However, owing to the complexity of BW resistance, the identification of the genomic regions controlling BW resistance is poorly understood and still remains to be unraveled in the pepper cultivars. In this study, we performed the quantitative trait loci (QTL) analysis to identify genomic loci and alleles, which play a critical role in the resistance to BW in pepper plants. The disease symptoms and resistance levels for BW were assessed by inoculation with *R. solanacearum*. Genotyping-by-sequencing (GBS) was utilized in 94 $F_2$ segregating populations originated from a cross between a resistant line, KC352, and a susceptible line, 14F6002-14. A total of 628,437 single-nucleotide polymorphism (SNP) was obtained, and a pepper genetic linkage map was constructed with putative 1550 SNP markers via the filtering criteria. The linkage map exhibited 16 linkage groups (LG) with a total linkage distance of 828.449 cM. Notably, QTL analysis with CIM (composite interval mapping) method uncovered *pBWR-1* QTL underlying on chromosome 01 and explained 20.13 to 25.16% by $R^2$ (proportion of explained phenotyphic variance by the QTL) values. These results will be valuable for developing SNP markers associated with BW-resistant QTLs as well as for developing elite BW-resistant cultivars in pepper breeding programs.

**Keywords:** pepper plant; *Ralstonia solanacearum*; single-nucleotide polymorphism (SNP); genotyping-by-sequencing (GBS); quantitative trait loci (QTL); pepper breeding

## 1. Introduction

Pepper plants (*Capsicum* spp.) derived from the regions of American tropics belong to the Capsicum genus and Solanaceae family, including peppers, paprika, tomatoes, eggplants, and potatoes. It is regarded as one of the most important vegetable crops worldwide owing to diverse positive aspects in field of cuisine, medicine and healthcare, and economy [1–3]. Pepper fruits are largely consumed as fresh and dried ingredients as well as processed foods and render a wide variety of essential bioactive elements, such as vitamins, minerals, phenolics, carotenoids, and capsaicinoids [4–8]. The consumption of pepper has been gradually increased for several decades, together with the cultivation area and production in agriculture (http://www.fao.org/faostat (accessed on 9 August 2021) [9]. On the basis of Food and Agriculture Organization (FAO) [9], the cultivation area has occupied around 4.5 million hectare, and pepper production has reached around 67 million

tons, including fresh and dried peppers, in the world [10]. Moreover, in terms of the world trade value of crops, the amount of chili peppers has ranked with the second position after tomato plants among the Solanaceae family [11]. However, pepper production is naturally threatened by biotic factors, including bacteria, fungi, and viruses in the agronomic field [12]. In particular, bacterial wilt (BW) is one of most destructive diseases throughout the world and has been widely spread in pepper crops across all over the Asia [13–15]. In 2017, BW led to a significant reduction of pepper yields and productions ranging from over 20 to less than 50% at most in the world cultivation area [16].

BW disease is caused by a soil-borne bacterial pathogen, *R. solanacearum,* and it is one of the global plant diseases [17]. The BW is seriously harmful for a large amount of the solanaceous family, including the vegetable crops of chili and sweet peppers, paprika, tomatoes, potatoes, and eggplants, which cause a plant-wilting disease. It is recognized as a wide range of hosts by invading over 450 different plant species through the broad climate spectrums containing tropical, subtropical, and temperate regions [18–20]. The pathogen of *R. solanacearum* enters plants via the natural opening and the wounded layers at the emergence sites of secondary roots or at root tips, which immigrate and colonize the host root cortex [21,22]. Subsequently, the *R. solanacearum* infects the parenchyma of the plant vascular system. The success of the invasion into xylem causes plant pathogenicity, including high population of the increased bacterial cells, diverse enzymes, and viscous di-and poly-saccharides by *R. solanacearum*. As such, the xylem vessels in plant roots are filled and blocked by the pathogenicity [23,24]. Interestingly, several reports have studied that the resistant cultivars possess an ability to restore the xylem transport system directly after the bacterial attacks, whereas the susceptible cultivars are observed with the xylem blocked by the occlusion derived from bacteria [25,26], leading to the damage of the water flow system inside the plant's xylem. The malfunction results from plant yellowing, wilting, and dying depending on the severity of the disease symptoms [25].

To date, multiple management methods to govern the BW disease have been developed and applied in agriculture. Indeed, the effects of combatting the devastating BW have been shown with agronomical, physical, chemical, and cultural methods, including the crop rotation of different types, utilization of bactericides, and plant breeding programs [26]. However, the management strategies have been reported with limited and insufficient effects on the BW disease regulation owing to the wide variety of host range, diverse genetic variations of the pathogen, and long-term survival in plants [26–28].

In general, it is considered that the most effective control method is to breed elite, resistant cultivars in the pepper crops against the BW [29]. Remarkably, a wide variety of BW resistant-pepper accessions have been determined. For example, the BW resistant-pepper accessions (*Capsicum* spp.) with LS2341, PI358812, Kerting, PI322726, PI322727, PI369998, PI377688, PI322728, Jatilaba, MC4, MC5, PBC 066, PBC 437, PBC 631, and PBC 1347 display high BW resistance against a wide array of BW pathogens [30–33]. In addition to this, some researches have studied that BW resistance is involved in a quantitative inheritance and is polygenically governed by multiple genes ($\geq$2 genes) in the pepper cultivar Mie-Midori [29]. A pepper cultivar, PM687, has been determined to have additive effects, which are influenced by the involvement of 2 to 5 candidate genes to regulate the BW resistance [34]. Additionally, A pepper accession called LS2341 has been identified with polygenes and linkage to a major quantitative trait loci (QTL), *Bw1,* located on chromosome 08, which possesses putative 44 candidate-resistance genes against *R. solanacearum* [35]. A recent report has uncovered a marker ID10-194305124 on the major QTL *qRRs-10.1* on chromosome 10, which consists of five candidate R genes containing putative leucine-rich repeat (LRR) receptor or NB-ARC proteins and three defense-associated genes in the resistance pepper cultivar BVRC1 [36].

QTL mapping is a basic and powerful tool for genetic investigation of quantitative traits and high-density linked markers. Although the conventional QTL mapping is a time-consuming, labor-intensive, and costly procedure [37], advances in next-generation sequencing (NGS) technologies reduce sequencing cost and contribute to the rapid iden-

tification of QTL and the assessment of genome-wide single nucleotide polymorphism (SNP) in pepper crop [38]. The utilization of NGS for SNP discovery is beneficial, as it generates a large amount of sequence data that can be used for genotype-phenotype association [37,39,40]. Genotyping-by-sequencing (GBS) technology is a simple and robust method that is practical as a high-throughput genotyping tool for a large number and huge amount of DNA as well as for complex genomes of crop species [41,42]. Furthermore, GBS is a cost-effective technique with the advantages of reduced sampling time, decreased sequencing cost, and no limited reference genome sequences [37,43]. Recent studies have reported that the GBS tool is successfully applied to the various crops, including chickpea, maize, wheat, onion, soybean, pepper, and rice, for QTL mapping, high-density genetic mapping, SNP discovery, GWAS, genomic selection (GS), and genotyping [44–52]. Since the GBS tool possesses a wide applicability of QTL identification in pepper (*C. annuum*), genetic analysis of quantitative traits and high-resolution-linked markers for BW would contribute to more accurate marker-assisted selection (MAS) in plant genetics and breeding via GBS.

In this study, we produced 94 $F_2$ recombinant lines derived from a cross between a resistant source of *C. annuum*, KC352, and a susceptible source of *C. annuum*, 14F6002-14, for QTL mapping of BW resistance to *R. solanacearum* isolates. Next, we constructed a genetic map with 94 $F_2$ recombinant offspring. High-resolution SNP markers associated with BW resistance revealed novel QTL regions on chromosome 01 via GBS. The result will be utilized for developing SNP markers involved in BW resistance and for selecting and breeding elite BW-resistant pepper plants.

## 2. Materials and Methods

### 2.1. Plant Materials and Growth Conditions

The parental lines of *C. annuum* KC352 and 14F6002-14 were provided by pepper and breeding institute (Gimje, Korea), respectively. A total of 94 $F_2$ individual lines were derived from a cross between KC352 and 14F6002-14. The pepper plants used for the experiment were grown at National Institute of Horticultural and Herbal Science (NIHHS, Wanju, Korea, 35°83′ N, 127°03′ E) under glasshouse conditions where temperature was controlled to 26/18 °C (day/night), and relative humidity (RH) was within 60–70%. The soil in pots was prepared as previously described in [53]. In brief, the commercial media (Bio Sangto, Seoul, Korea) was composed of coco peat (47.2%), peat moss (35%), vermiculite (10.0%), zeolite (7%), dolomite (0.6%), humectant (0.006%), and fertilizers (0.194%, 270 mg kg$^{-1}$ N, P, and K), respectively.

### 2.2. DNA Extraction

A total of 96 plant samples from 9–10 leaf stage of 4-week-old plants were ground with the TissueLyser II (Qiagen, Hilden, Germany), and genomic DNA (gDNA) from the samples was extracted using cetyl trimethyl ammonium bromide (CTAB) method as previously described in [54].

### 2.3. Disease Assay and Resistance Index Scoring

To evaluate resistance against *R. solanacearum*, resistance screening was conducted in 45–48 $F_2$ offspring derived from a cross between parental lines of KC352 and 14F6002-14 with three independent replicates (Supplementary Figure S1). A total of 94 $F_2$ lines were tested for the resistance screening and subsequent GBS analysis. To prepare the inoculum, the *R. solanacearum* of WR-1 strain isolates (race 1, biovar 3) were cultivated on NA medium and incubated at 28 °C for bacterial cell growth for 2–3 days. The plates were collected with distilled water to harvest the cells. The concentration was determined by measuring OD at 600 nm = 0.3, and the cell density was adjusted to approximately $10^7$–$10^8$ cfu per mL before inoculation. Two parental lines and 94 $F_2$ offspring were inoculated at the 6–7 leaf stage onto the plant roots with 5 mL of the bacterial suspension after wounding the plant roots by stabbing a scalpel along with two sides at a soil depth of 1–2 cm. The inoculated

plants were kept under vinyl-protected conditions at 25 to 30 °C, and disease resistance was continuously observed and recorded after inoculation. *C. annuum* KC352 was utilized as the resistant control, whereas *C. annuum* 14F6002-14 was utilized as the susceptible control to compare the severity of disease symptoms in $F_2$-segregating populations. The disease symptoms and disease resistance index were evaluated on the basis with disease scale of 0–4 as previously described in [25], where 0 = no visible symptoms, 1 = 1 to 25% of wilted leaves, 2 = 26 to 50% of wilted leaves, 3 = 51 to 75% of wilted leaves, and 4 = 76 to 100% of wilted leaves.

*2.4. Preparation of Libraries for Genotype-by-Sequencing (GBS) Analysis*

A total of 96 individuals were subjected to GBS analysis. The quantity and quality of extracted gDNAs were validated using 1% agarose gel electrophoresis before running next-generation sequencing (NGS). The preparation of GBS libraries was conducted as provided by SEEDERS sequencing company (Daejeon, Korea). To construct GBS libraries, gDNAs were digested with *Ape*KI (New England Biolabs, Ipswitch, MA, USA) [41] with a minor modification. In detail, oligonucleotides for the top and bottom strands of each barcode adapter and a common adapter were separately diluted in 50 μM TE buffer and annealed in thermocycler conditions followed with 95 °C, 2 min (ramp down to 25 °C by 0.1 °C/s; 25 °C, 30 min; 4 °C hold). Barcodes and common adapters were diluted in 10× adapter buffer, including 500 mM NaCl and 100 mM Tris-Cl to 10 μM, and 2.4 μL of the mixture was applied into a 96-well PCR plate. Then, 100 ng/μL of DNA samples were added to individual adapter-containing wells and digested for overnight at 75 °C with 3.6 U ApeKI (New England Biolabs, Ipswitch, MA, USA) in 20-μL volumes. Adapters were then ligated to sticky ends by adding 30 μL of a mixture containing 10× ligase buffer and 200 unit of T4 DNA ligase (MG Med, Seoul, Korea) to individual wells. The samples were incubated at 22 °C for 2 h and heated to 65 °C for 20 min to remove the activity of the T4 DNA ligase. The 96 digested DNA samples possessing a different barcode adapter were combined with each 5 μL and were purified using a purification kit (QIAquick PCR Purification Kit; Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Restriction fragments from each library were then amplified in 50-μL volumes containing 2 μL pooled DNA fragments, Herculase II Fusion DNA Polymerase (Agilent, Santa Clara, CA, USA), and 25 pmol with each of the primers in [41]. Polymerase chain reaction (PCR) was conducted with the following conditions: one cycle at 95 °C for 2 min, 16 cycles at 95 °C for 30 s, 62 °C for 30 s, 68 °C for 30 s, and stopped at 68 °C for 5 min. The amplified fragment size and library quality were assessed with Agilent Tape station with high-sensitivity DNA chip. Whole-genome sequences were conducted using Illumina Hiseq X ten platform (Illumina, San Diego, CA, USA).

*2.5. Sequencing, Alignment, and SNP Genotyping*

Raw reads were de-multiplexed, and the barcode sequences were trimmed using SEEDERS in-house python script as previously described in [49]. Reads were also trimmed using the Cutadapt (ver. 1.8.3) to eliminate the sequences of the common adapter. The de-multiplexed reads were processed by the SolexaQA package ver.1.13 [41]. Next, bad-quality bases with low Phred quality score (Q = 20 or 0.05 probability of error) were trimmed using DynamicTrim in the SolexaQA package, and the read lengths lower than 25 bp with poor-quality sequence were discarded, using Lengthsort program in the SolexaQA package [55]. The processed and cleaned reads were applied to align with *C. annuum* cv. CM334 reference genome (ver. 1.55, http://www.sgn.cornell.edu/ (accessed on 19 November 2019), and the read depth was counted by the number of aligned reads via the Burrows–Wheeler Aligner (BWA, 0.6.1-r104) program as described in [56]. The BWA was carried out with following default options: gap open penalty ($-O$) = 15, number of threads ($-t$) = 16, mismatch penalty ($-M$) = 6, maximum differences in the seed ($-k$) = 1, and gap extension penalty ($-E$) = 8, except for seed length ($-l$) = 30. The detection of raw SNPs and the consensus sequences were acquired from the resulting mapped reads with BAM format file using

SAMtool (v.0.1.16) utilities [57]. For SNP calling, the varFilter command in the SAMtool was utilized with default options as previously described in [17,49]. Finally, on the basis of ratio of SNP/InDel reads in the mapped reads, variant types of SNP were grouped with three categories: homozygous SNP/InDel for read rate $\geq$90%, heterozygous SNP/InDel for read rate $\geq$40% and $\leq$60%, and the rest defined as "etc." [49,58,59].

### 2.6. Linkage Map Construction

Genetic linkage maps of $F_2$ segregating lines were illustrated using the JoinMap ver. 4.0 (Kyazma B.V., Wageningen, The Netherlands). A total of 1550 SNPs were grouped into 16 linkage groups (LGs) with a logarithm of the odds (LOD) threshold score $\geq$5.5, and a maximum distance of 30 centiMorgans (cM) were used. The genetic map distance of the SNP markers was converted to cM using the Kosambi's mapping function [60]. To remove the skewed SNP and the segregation distortion, the chi-square test ($p < 0.001$) was applied, and the SNP markers were filtered with identical segregation or missing rate $\geq$30%. Final genetic linkage maps of KC352 and 14F6002-14 with the 1550 SNPs were drawn using MapChart ver. 2.3 software [61].

### 2.7. QTL Analysis and Candidate Genes Prediction

QTL analysis was performed with composite interval mapping (CIM) to map the QTLs involving pepper bacterial wilt resistance using the Windows QTL Cartographer v. 2.5 program [62]. The CIM was operated at a 1.0-cM walk speed using the model 6 parameters (standard model) and the forward and backward regression model. The LOD threshold level for significance of each QTL was determined as 1000 permutations of $p < 0.05$. To identify candidate genes, the positions of highly significant QTLs regions on the genetic map were compared with their physical positions on the *C. annuum* cv. CM334 reference genome (ver. 1.55, http://www.sgn.cornell.edu/ (accessed on 19 November 2019), and 1 Mb left and right sequences were mined for candidate genes from respective corresponding marker. Putative functions of the candidate genes were further annotated with an application of sequence alignment using CM334 reference genome (ver. 1.55, https://solgenomics.net/ (accessed on 21 December 2021), Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.genome.jp/kegg/ (accessed on 21 December 2021), SwissProt (https://www.uniprot.org/ (accessed on 21 December 2021), Gene Ontology (GO) (https://www.geneontology.org/ (accessed on 23 December 2021), and the NCBI non-redundant protein (NR) (https://ncbi.nih.gov/blast/db/ (accessed on 23 December 2021) with default values.

### 2.8. Data Analysis

The Tukey's HSD/Kramer test ($p < 0.05$) and the descriptive statistics of disease index were analyzed using SPSS program (IBM SPSS v27.0, Chicago, IL, USA). The Pearson's correlation coefficients were calculated using the R statistical Software (ver. 4.0.1, https://www.r-project.org (accessed on 3 January 2022)).

## 3. Results

### 3.1. Phenotyping of the Resistance for Bacterial Wilt Disease

In order to evaluate bacterial wilt (BW) resistance, we observed phenotypes for 5–21 days after inoculation of *R. solanacearum* suspension into parental lines and 94 $F_2$ lines. The degree of disease severity was evaluated as disease index (DI), with disease scale ranging from 0 (no symptoms) to 4 (76 to 100% wilted leaves) (Figure 1A). Among the 94 inoculated plants, 16 plants were observed with no visible symptoms (DI = 0), representing a resistant line to BW, whereas 49 plants were observed with 76–100% wilted leaves (DI = 4), representing a susceptible line to BW (Figure 1B). In addition to this, we observed 14 plants with 1 to 25% wilted leaves (DI = 1), 7 plants with 26 to 50% wilted leaves (DI = 2), and 8 plants with 51 to 75% wilted leaves (DI = 3) (Figure 1B). Overall, the average DI value of the $F_2$ population was 2.638, and the wilt rate (%) was 68.085. The skewness and kurtosis

value of the DI was $-0.604$ and $-1.352$, respectively, suggesting that the resistance level of the plants to *R. solanacearum* is susceptible, and the population might be a non-normal distribution rather than a normal distribution (Table 1).
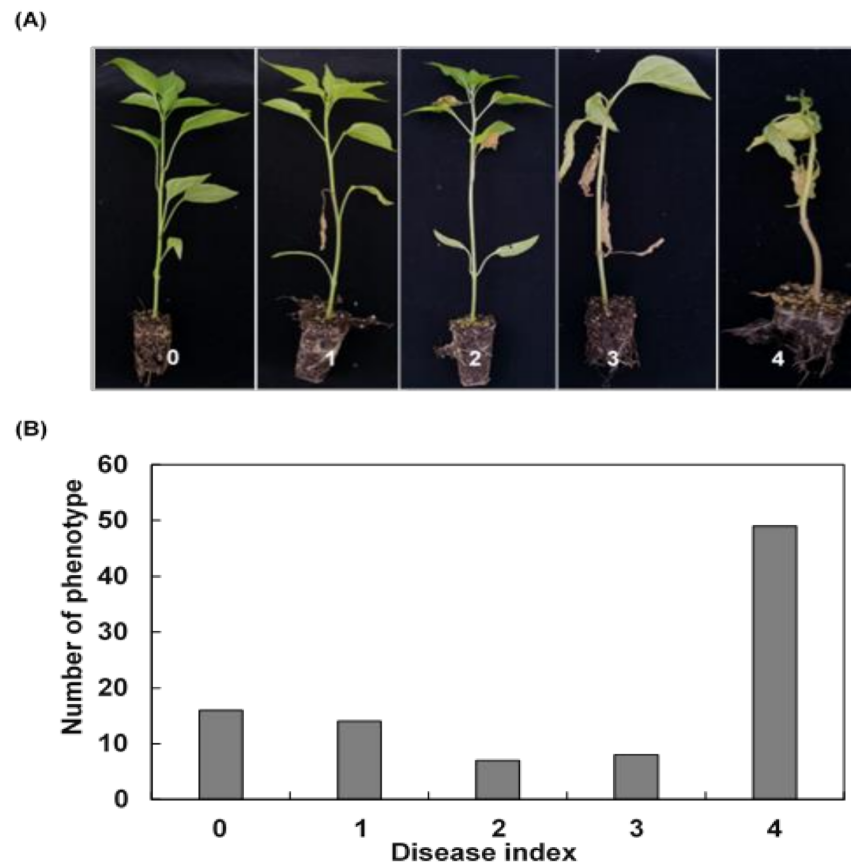


**Figure 1.** Distribution of the disease index after inoculation with *R. solanacearum*. (A) disease index was classified into disease scales of 0–4, where 0 = no visible symptoms, 1 = 1 to 25% wilted leaves, 2 = 26 to 50% wilted leaves, 3 = 51 to 75% wilted leaves, and 4 = 76 to 100% wilted leaves, and (B) disease index (DI) was recorded in $F_2$-segregating populations, including 94 genotypes from the cross between a resistant parent (KC352) and a susceptible parent (14F6002-14).

**Table 1.** Descriptive statistics of disease index.

| Trait | $F_2$ Population (*n* = 94) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Max** | **Min** | **Range** | **Wilt Rate (%)** [a] | **Average** [b] | **Standard Error** | **Variation** | **Skewness** | **Kurtosis** |
| Disease index | 4 | 0 | 0–4 | 68.085 | 2.638 | 0.167 | 2.620 | −0.604 | −1.352 |

[a] Wilting rate was defined as DI that was more than 2. [b] Average was calculated on the basis of 0–4 rating scale as follows: DI = ∑(disease score x the number of plants corresponding to each disease score)/total number of plants.

### 3.2. GBS Analysis

Next, in order to conduct a genotyping-by-sequencing (GBS) analysis, all 96 plants were collected, and a construction of 96-plex GBS library was generated. As such, a total of approximately 108.0 Gbp of DNA sequences (715,257,004 reads) were obtained from single-lane sequencing using Illumina Hiseq X ten platform (Table S1). The GBS raw data ere de-multiplexed according to the 96 barcode sequences. The de-multiplexed sequences of the 96 samples were trimmed by eliminating the sequences of the barcode and adaptor and removing the low-quality information. Finally, the average number and total length of trimmed reads were 6,046,776 and 696 Mbp, respectively. In addition, the average length of trimmed reads (bp) and the total trimmed raw data were 119.99 bp and

94.45%, respectively (Table S1). The trimmed data were further mapped to the reference genome: *Capsicum. annuum* cv. CM334 ver. 1.55, sourced by Sol Genomics Network (http://www.sgn.cornell.edu/ (accessed on 19 November 2019). The average numbers of mapped reads and mapped regions were 5,216,671 and 125,718, respectively (Table S1). The average depth and length of the mapped regions were 14.75 and 233.34 bp, which covered 0.56% of the reference genome. To further mine SNPs from the sequence data, in-house GBS analysis pipeline was applied with filtering criteria. A total of 628,437 raw SNPs was identified in 94 $F_2$ lines (Table S2). The SNPs were filtered to identify putative markers using the criteria of 30% missing values across the genotyped individual and MAF $\geq$ 25%, which yielded a total of 146,217 SNPs. Moreover, 11,020 SNPs were filtered using both missing <30% and MAF > 25% condition. Furthermore, 4387 homozygous SNPs in KC352 were selected (Table S2). After generating a map of the genotyping and SNP selections using 500 kb as the window size, 1643 SNP markers were identified, and a total of 1639 SNPs were produced with polymorphic SNP between KC352 and 14F6002-14 as the parents (Table S2).

*3.3. Construction of Linkage Mapping*

In order to construct a pepper genetic linkage map, 1639 SNPs were utilized for linkage grouping using the SNP matrix from GBS analysis. As such, the linkage map consisted of 1550 SNP markers on 16 linkage groups (LG) (Table 2 and Figure 2). The linkage map covered a total length of 828.449 cM with an average distance of 0.676 cM between adjacent markers (Table 2 and Figure 2). Next, LG_04 showed the maximum lengths, which were 78.045 cM (chromosome 04) in the largest LG, whereas LG_05-2 showed the minimum lengths, which were 15.952 cM (chromosome 05) in the smallest LG (Table 2). The number of mapped SNPs per chromosome ranged from the minimum 11 in chromosome 10 to the maximum 172 in chromosome 03, with an average number of 96.875 SNP markers per LG (Table 2 and Figure 2). Moreover, the correlation coefficient between genetic and physical maps was estimated among the 16 linkage groups. Chr.07 exhibited the highest correlation coefficient of 0.876, and the average correlation coefficient was 0.670.

**Table 2.** Summary of the pepper genetic linkage map constructed using SNP markers derived from genotyping-by-sequencing (GBS) analysis for bacterial wilt (BW) disease resistance. The higher Pearson's correlation coefficient indicates the closer correlation.

| Chromosome | Linkage Group | Number of SNP Marker | Genetic Distance (cM) | Correlation Coefficient |
|---|---|---|---|---|
| Chr.01 | LG_01-1 | 123 | 82.134 | 0.755 |
|  | LG_01-2 | 49 | 38.254 | 0.385 |
| Chr.02 | LG_02-1 | 60 | 20.799 | 0.096 |
|  | LG_02-2 | 90 | 70.704 | 0.644 |
| Chr.03 | LG_03 | 172 | 59.561 | 0.790 |
| Chr.04 | LG_04 | 117 | 78.045 | 0.867 |
| Chr.05 | LG_05-1 | 91 | 47.582 | 0.840 |
|  | LG_05-2 | 12 | 15.952 | 0.437 |
| Chr.06 | LG_06 | 146 | 52.635 | 0.867 |
| Chr.07 | LG_07 | 136 | 61.809 | 0.876 |
| Chr.08 | LG_08 | 54 | 47.681 | 0.513 |
| Chr.09 | LG_09 | 114 | 74.982 | 0.786 |
| Chr.10 | LG_10-1 | 11 | 19.247 | 0.603 |
|  | LG_10-2 | 139 | 54.224 | 0.857 |
| Chr.11 | LG_11 | 123 | 59.579 | 0.867 |
| Chr.12 | LG_12 | 113 | 45.261 | 0.541 |
| Total | 16 | 1550 | 828.449 | - |
| Average | - | 96.875 | 51.778 | 0.670 |
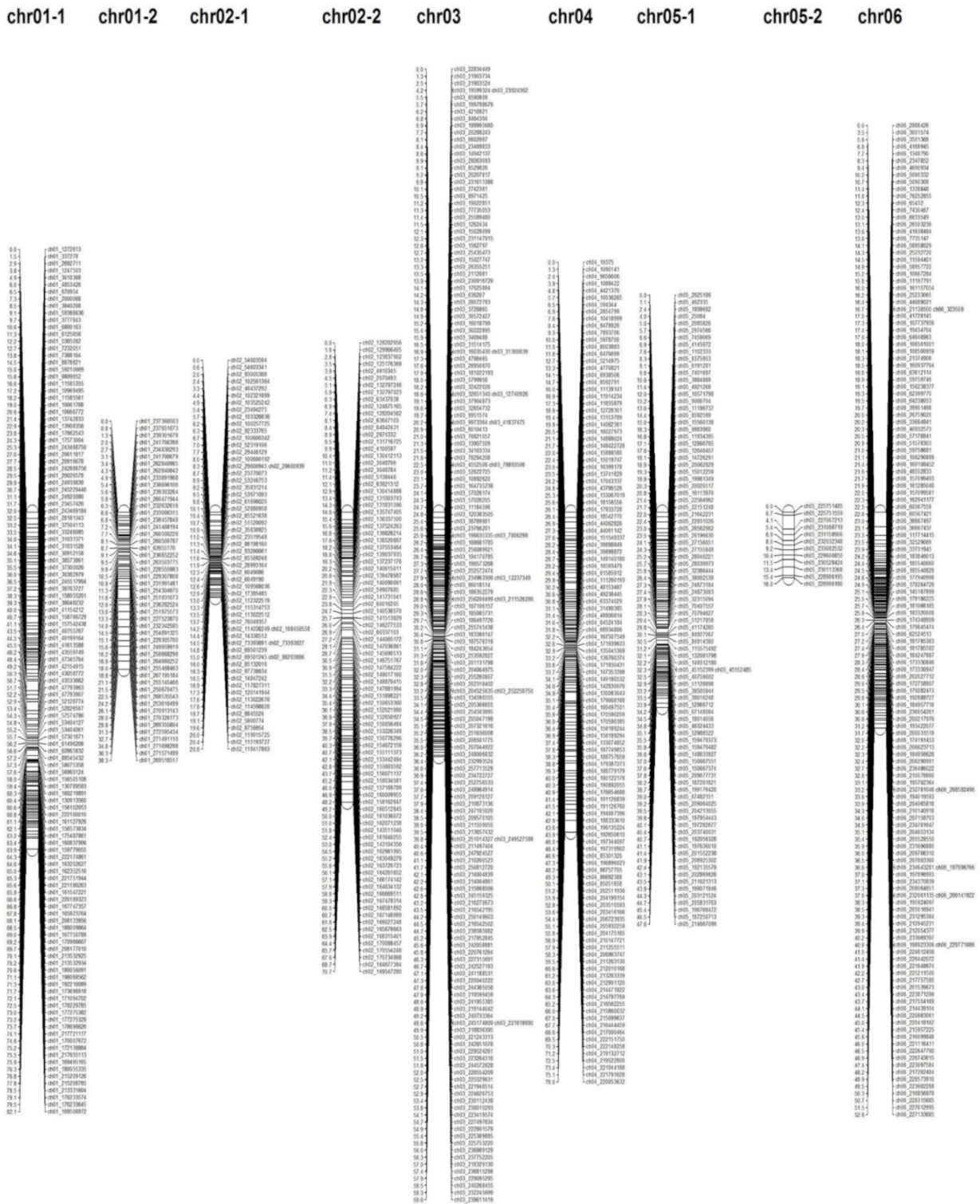
**Figure 2.** *Cont.*
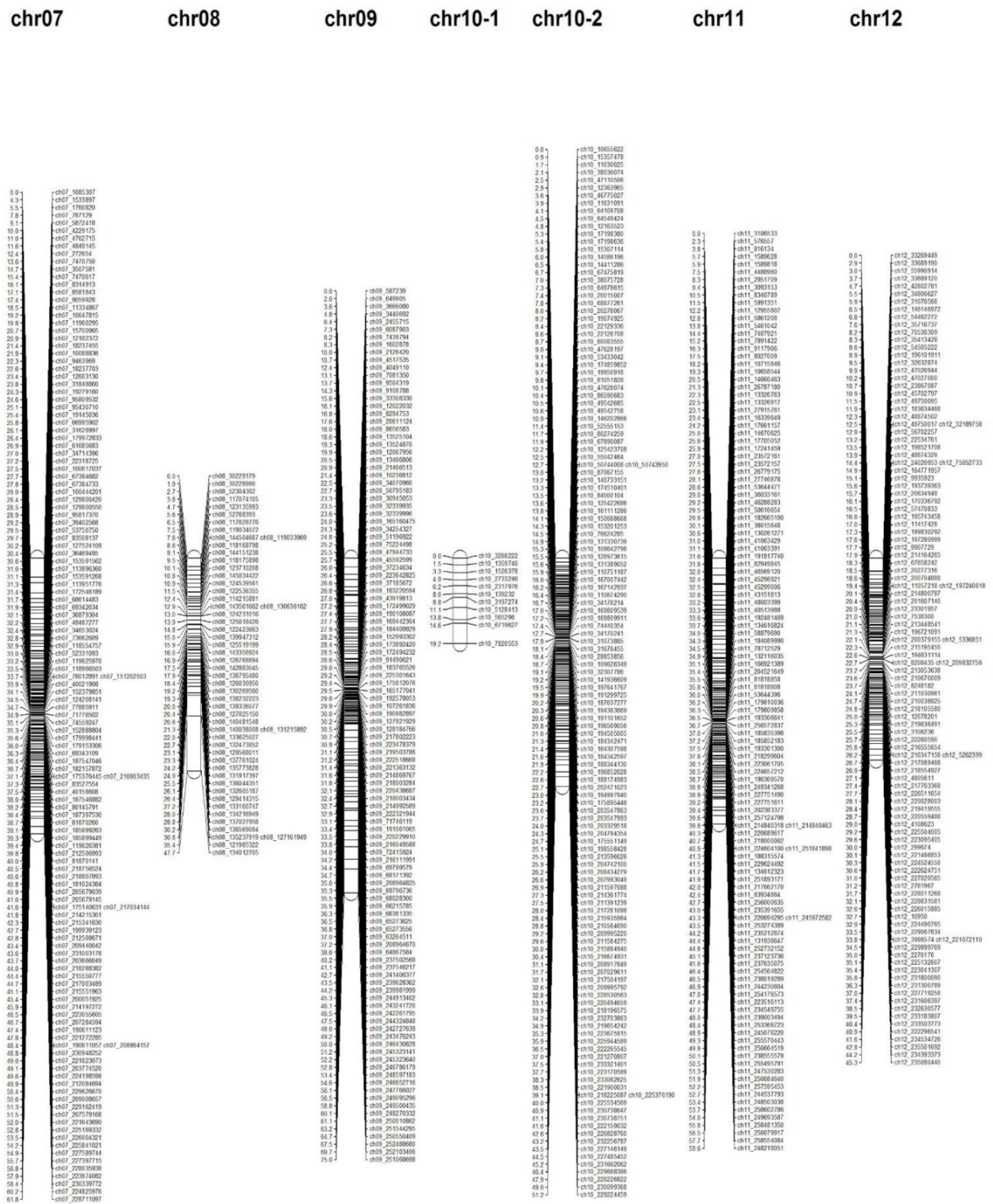
**Figure 2.** Distribution of single-nucleotide polymorphism (SNP) markers on 16 linkage groups of F$_2$ pepper population. The pepper genetic linkage map consisted of 1550 SNP markers derived from genotyping-by-sequencing (GBS) analysis. SNP names were shown on the right side of the linkage map and genetic distances (cM) between SNPs on the left.

### 3.4. QTL Analysis for Bacterial Wilt (BW) Resistance

In order to analyze significant quantitative trait loci (QTL) regions, the 1000 permutation was tested at $p < 0.05$, and the LOD threshold was calculated as 5.5 by QTL cartographer. Notably, the LOD values ranged from 5.69 to 7.06, which was detected in chromosome 01 via CIM (Figure 3A). On the basis of the threshold levels, we designed one QTL, *pBWR-1*, named the pepper Bacterial Wilt Resistance-1 on chromosomes 01 (Figure 3B). In addition, the significant LOD $\geq 5.5$ regions identified at the *pBWR-1* QTL region were within LG_01-1 on chromosome 01. The *pBWR-1* QTL was located between the ch01_47793963 and ch01_161127926 markers on chromosome 01 with twelve markers (Figure 3B and Table 3). The explained phenotypic variance of the QTL was ranged from 20.13 to 25.16% (Table 3).
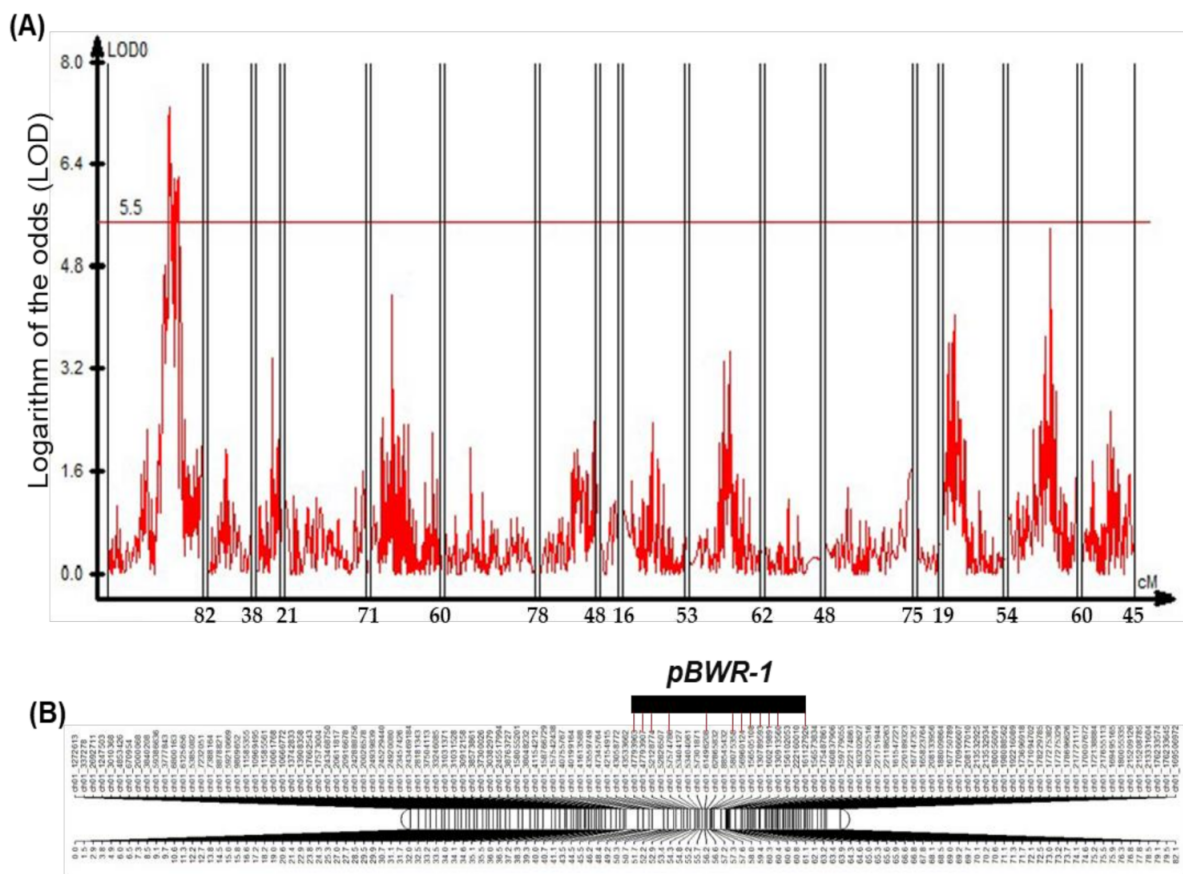


**Figure 3.** (**A**) Quantitative trait loci (QTL) plot. QTL associated with bacterial wilt (BW) disease resistance in the $F_2$ population derived from the parental lines of KC352 and 14F6002-14 in the linkage groups obtained via CIM. The LOD threshold is indicated by the red-colored horizontal line. (**B**) Physical map of chromosome 01 with SNP markers used for the mapping of BW resistance locus as shown in Figure 2. The QTL positions (LOD $\geq 5.5$) and SNP markers of BW resistance are indicated with a black bar combined with red-colored vertical lines above the linkage map.

**Table 3.** Summary of significant QTLs (LOD ≥ 5.5) regions with composite interval mapping (CIM) analysis.

| Chr. | Marker (Chr_pos) | QTL Position (cM) | LOD | Additive | Dominant | [a] $R^2$ (%) | [b] \|d/a\| | [c] \|d/a\| Value | [d] Number of Gene |
|---|---|---|---|---|---|---|---|---|---|
| | ch01_47793963 | 51.73 | 7.065493 | −0.85811 | 0.701208 | 23.9114 | D | 0.82 | 1 |
| | ch01_47793907 | 52.21 | 7.323219 | −0.88307 | 0.690647 | 25.1692 | PD | 0.78 | 1 |
| | ch01_52128774 | 52.94 | 6.962209 | −0.88383 | 0.603339 | 24.0817 | PD | 0.68 | 5 |
| | ch01_57574786 | 54.32 | 6.440147 | −0.85645 | 0.651621 | 23.1222 | PD | 0.76 | 0 |
| | ch01_61496208 | 56.25 | 6.180448 | −0.89693 | 0.496732 | 24.5181 | PD | 0.55 | 2 |
| Chr. 01-1 | ch01_58075358 | 57.31 | 5.682495 | −0.79976 | 0.700647 | 20.9856 | D | 0.88 | 0 |
| | ch01_56960124 | 57.65 | 5.983697 | −0.93776 | 0.399064 | 23.0298 | PD | 0.43 | 0 |
| | ch01_156505108 | 58.98 | 6.161348 | −0.88705 | 0.631756 | 24.0344 | PD | 0.71 | 11 |
| | ch01_130789593 | 59.46 | 5.602784 | −0.81563 | 0.56539 | 20.171 | PD | 0.69 | 0 |
| | ch01_160219891 | 60.27 | 5.506165 | −0.95201 | 0.162341 | 22.5886 | A | 0.17 | 2 |
| | ch01_130913560 | 60.36 | 6.216155 | −0.92824 | 0.283328 | 22.2838 | PD | 0.31 | 0 |
| | ch01_161127926 | 61.1 | 5.692397 | −0.85691 | 0.394691 | 20.1396 | PD | 0.46 | 10 |

[a] $R^2$, proportion of phenotypic variance explained by a major QTL; [b] \|d/a\|, estimation of gene action; A, (additive effect) 0–0.20; PD, (partial dominance) 0.21–0.80; [c] D, (dominance) 0.81–1.20; OD, (overdominance) >1.20. [d] Number of gene was selected with the 1 Mb to the left and right of the corresponding marker.

*3.5. Prediction and Annotation of Candidate Genes*

In order to identify candidate genes within the major QTL region, the number of genes was selected with the 1 Mb to the left and right of the corresponding markers, and a total of 31 candidate genes were annotated on the basis of CM334 reference genome, Swiss-Prot, and the NCBI non-redundant protein (NR) databases (Table 4). In addition, functional classification of 31 predicted genes was further analyzed along with the Kyoto Encyclopedia of Genes Genomes (KEGG) pathway and Gene Ontology (GO) term (Table 4). As such, the analysis of GO term enrichment identified one gene (CA01g20110) annotated with "defense response" (GO: 0006952), and the KEGG analysis identified one gene (CA01g20130) with "plant–pathogen interaction" (KO: 13457). Overall, four of the 31 candidate genes were assigned to defense-associated genes; CA01g18650 in ch01_156505108 marker and CA01g20130 (putative disease resistance protein RPM1), CA01g20110 (Thaumatin-like protein), and CA01g20140 (NB-ARC domain-containing protein) in ch01_161127926 marker were predicted as disease-resistance proteins. Taken together, the annotated four disease-resistance/defense-associated genes would be crucial candidate genes for *pBWR-1* QTL in the present study.

**Table 4.** The candidate genes within the significant QTL regions.

| Gene Name | Gene Start | Gene End | CM334(v.1.55) | KEGG/GO | SwissProt | NR |
|---|---|---|---|---|---|---|
| CA01g12080 | 47862177 | 47863580 | Putative amino acid transporter | - | Putative polyamine transporter | Putative polyamine transporter |
| CA01g12450 | 52035941 | 52041393 | Calcium-dependent protein kinase 17 | K13412 (calcium-dependent protein kinase) | Calcium-dependent protein kinase 17 | Calcium-dependent protein kinase 17-like |
| CA01g12460 | 52113432 | 52115689 | Rho GTPase-activating protein | - | Rho GTPase-activating protein 7 | rho GTPase-activating protein 7 |
| CA01g12470 | 52119767 | 52121954 | Putative rho GTPase activator | - | Rho GTPase-activating protein 7 | PREDICTED: rho GTPase-activating protein 7-like |
| CA01g12480 | 52128303 | 52129214 | Rho GTPase-activating protein 7-like isoform X2 (*Solanum tuberosum*) | - | Rho GTPase-activating protein 7 | PREDICTED: rho GTPase-activating protein 7-like |
| CA01g12490 | 52147172 | 52148002 | Phospholipase A22 | - | Uncharacterized protein | Putative rho GTPase-activating protein 7-like isoform X1 |
| CA01g13370 | 61438907 | 61440480 | Arsenite-resistance protein, putative | - | Serrate RNA effector molecule | Serrate RNA effector molecule |
| CA01g13380 | 61446486 | 61447578 | Arsenite-resistance protein, putative | - | Serrate RNA effector molecule | Serrate RNA effector molecule |
| CA01g18610 | 156433050 | 156436475 | Detected protein of unknown function | - | O-fucosyltransferase family protein | Putative siroheme synthase-like |
| CA01g18620 | 156438428 | 156442076 | AT5g40850/MHK7_8 | K02303 (uroporphyrin-III C-methyltransferase) | Siroheme synthase | PREDICTED: siroheme synthase |
| CA01g18630 | 156454093 | 156456205 | Putative glutathione S-transferase T1 | K00799 (glutathione S-transferase) | Putative glutathione S-transferase | PREDICTED: probable glutathione S-transferase |
| CA01g18640 | 156457355 | 156460628 | Cohesin subunit rad21, putative | K06670 (RAD21; cohesin complex subunit SCC1) | Uncharacterized protein | PREDICTED: sister chromatid cohesion 1 protein 3 |
| CA01g18650 | 156487695 | 156490046 | Anthranilate phosphoribosyltransferase-like protein | - | Uncharacterized protein | Putative disease-resistance protein RPM1-like |
| CA01g18660 | 156497587 | 156514448 | DNA-directed RNA polymerase | - | DNA-directed RNA polymerase subunit beta | PREDICTED: DNA-directed RNA polymerase I subunit 2 |

**Table 4.** *Cont.*

| Gene Name | Gene Start | Gene End | CM334(v.1.55) | KEGG/GO | SwissProt | NR |
|---|---|---|---|---|---|---|
| CA01g18670 | 156515197 | 156518484 | Putative synaptotagmin | - | Protein QUIRKY | Protein QUIRKY |
| CA01g18680 | 156539078 | 156540145 | Detected protein of unknown function | K22733 (NIPA, SLC57A2S, magnesium transporter) | Magnesium transporter | Putative magnesium transporter NIPA8 |
| CA01g18690 | 156550800 | 156555986 | DnaJ-like protein | K03686 (DnaJ, molecular chaperone DnaJ) | Uncharacterized protein | Hypothetical protein T459_02496 |
| CA01g18700 | 156563245 | 156580138 | Eukaryotic translation initiation factor 3 subunit, putative | K03255 (TIF31, CLU1, protein TIF31) | Clustered mitochondria protein | Clustered mitochondria protein |
| CA01g18710 | 156602485 | 156602895 | Detected protein of unknown function | K11253 (Histone H3) | Histone H3 | PREDICTED: histone H3.2-like |
| CA01g19920 | 160217017 | 160229898 | Ankyrin repeat family protein (*Populus trichocarpa*) | K24810 (IBTK, inhibitor of Bruton tyrosine kinase) | Ankyrin repeat domain-containing protein | Hypothetical protein FXO37_07210 |
| CA01g19930 | 160315849 | 160316316 | Polypeptide with a gag-like domain | - | LRRNT-2 domain-containing protein | Uncharacterized protein |
| CA01g20080 | 161050160 | 161051870 | Transcription factor bHLH130-like isoform X1 (*Citrus sinensis*) | - | Transcription factor bHLH130 | PREDICTED: transcription factor bHLH130-like |
| CA01g20090 | 161051871 | 161052295 | DNA binding protein, putative | - | Transcription factor bHLH130 | PREDICTED: transcription factor bHLH130-like |
| CA01g20100 | 161113518 | 161117641 | Ras-related protein RGP1-like (*Solanum tuberosum*) | K07904 (RAB11A, Ras-related protein Rab-11A) | Ras-related protein RABA4c | Ras-related protein RABA4c |
| CA01g20110 | 161118723 | 161120113 | Thaumatin-like protein SE39b | -/GO (0006952): defense response | Uncharacterized protein | PREDICTED: thaumatin-like protein 1b isoform X1 |
| CA01g20120 | 161154079 | 161154489 | Histone H3 family protein (*Populus trichocarpa*) | K11253 (H3, histone H3) | Histone H3 | PREDICTED: histone H3.2-like |
| CA01g20130 | 161161494 | 161162582 | Disease resistance protein RPM1-like (*Fragaria vesca*) subsp. vesca | K13457 (RPM1, RPS3; disease-resistance protein RPM1) | Uncharacterized protein | Hypothetical protein T459_01449 |

**Table 4.** *Cont.*

| Gene Name | Gene Start | Gene End | CM334(v.1.55) | KEGG/GO | SwissProt | NR |
|---|---|---|---|---|---|---|
| CA01g20140 | 161184987 | 161187221 | Detected protein of unknown function | - | NB-ARC domain-containing protein | PREDICTED: putative late blight-resistance protein homolog R1B-23 |
| CA01g20150 | 161212363 | 161212704 | Detected protein of unknown function | - | Uncharacterized protein | Putative cellulose synthase A catalytic subunit 3 (UDP-forming)-like |
| CA01g20160 | 161215973 | 161218180 | Detected protein of unknown function | - | Uncharacterized protein | Uncharacterized protein LOC107839230 isoform X2 |
| CA01g20170 | 161221263 | 161222494 | Detected protein of unknown function | - | Uncharacterized protein | Uncharacterized protein LOC107839213 |

## 4. Discussion

BW is one of the most destructive pepper diseases worldwide, leading to the reduction of yield and production in pepper cultivation [16,63]. It is difficult to manage BW disease owing to a wide array of plant host range, a huge number of diverse BW isolates, and its long survivability in pepper plants [18–20]. Thus, it is indispensable to breed resistant pepper cultivars against BW. Although molecular marker-assisted selection (MAS) for BW resistance can contribute to a rapid selection of BW-resistant breeding in pepper crops, a few studies have determined QTL regions [17,36,62]. In this study, we performed QTL analysis to develop molecular markers that are associated with BW resistance in pepper (*Capsicum annuum*) by evaluating the 94 $F_2$ recombinant lines obtained by a cross between a resistant and a susceptible parental line. We first constructed a genetic linkage map using GBS approach and identified significant QTL regions on chromosome 01 associated with BW resistance.

### 4.1. Construction of Pepper Genetic Map

GBS is a genome-wide genotyping, powerful, and straightforward approach that takes advantage of enzyme-based genome analysis, thereby conferring a rapid and cost-effective analysis of the huge and complex genome in organisms. The GBS tool has been widely utilized in genotyping segregated plants via the combination of a high-throughput next-generation sequencing (NGS) technology to generate multiplexed libraries using barcoded adapters [64,65]. With the application of GBS analyses, it has been reported that a large amount of barely SNPs are produced, and ≥34,000 SNPs are mapped onto its reference genome sequences, and ≥20,000 wheat SNPs are constructed onto its reference map [65]. Moreover, the method has identified 9998 SNPs and 64,754 SNPs located on the peach and pea genomes, respectively [66,67]. Notably, recent studies have explored and evaluated 1,399,567 SNP in onion using a GBS library [50], and a total of 91,132 raw SNPs were uncovered via a QTL study involved in flowering time in perilla [49]. In addition to this, current applications of GBS analysis have exhibited a total of 22,446 SNPs for QTL mapping of the resistance against the cucumber mosaic virus in a cucumber crop as well as a total of 66,405 SNPs for *Phytophthora capsici* resistance in a pepper crop [48,59]. In the present study, a total of 628,437 raw SNPs were identified and successfully genotyped with 94 $F_2$ offspring using GBS. Around 108 Gbp of raw data (Tables S1 and S2) and a total 1639 SNPs were finally produced, and the SNP markers were shown with genome-wide distribution, covering the whole pepper genome (Figure 2). A total of 1550 SNP markers were ultimately constructed on a genetic linkage map, which comprised 16 LGs, including one linkage group on chromosome 03, 04, 06, 07, 08, 09, 11, and 12 as well as two linkage groups on chromosome 01, 02, 05, and 10, respectively (Figure 2 and Table 2). In general, SNP markers used for genetic mapping are based on the polymorphic markers of the parent. When the polymorphism of the parent is absent in a large region within the middle of the genome, separating two linkage groups on one chromosome can often be produced although the genetic map is physically one chromosome. In particular, the phenomena often occur when working with breeding lines. Indeed, previous reports have shown that Yellow lupin (*Lupinus luteus* L.) possesses 26 chromosomes, but 40 linkage groups were constructed for QTL mapping via NGS approaches [68], and a linkage group of LG01 was divided into two LGs on chromosome 01 in perilla via GBS [49]. Recently, it has been determined that linkage map is constructed with the resistance trait of powdery mildew from $F_5$ pepper population via GBS. The LG07 is separated into two linkage groups on chromosome 07 [69].

### 4.2. Genetic Inheritance of pBWR-1 QTL on Chromosome 01

As mentioned above, it has been reported that the genetic analysis and identification of resistance genes play a crucial role in the field of crop breeding against BW [24,30,31,34–36,38,63]. Nonetheless, the genetic inheritance is poorly understood in the involvement of BW resistance in pepper crops, and the mechanism of genetic inheritance is still unclear since different pepper sources result in different values of BW resistance. In our results, we

evaluated 49 F$_2$ offspring as the DI value 4 (the most severe symptoms, 76 to 100% wilted leaves), whereas 14 plants were evaluated as DI value 1 (no visible symptoms) with the comparison of the parental lines after BW inoculation (Figure 1). We observed susceptible plants 3.5 times more than resistant plants in the F$_2$ population, implying that BW resistance might be a partially recessive trait in the pepper lines used for our experiment. In similar line with our result, the genetic inheritance of BW resistance has been unraveled, and the resistance homozygous recessive (rr) allele was identified using F$_2$ populations derived from a cross between resistant Anugraha and susceptible Pusa Jwala of near-isogenic lines (NILs) in pepper crops [70]. On the contrary, it has been determined that the inheritance action of BW resistance is involved in an incomplete dominance with more than two BW-resistance genes using the progeny derived from a cross between the *capsicum* Mie-Midori (resistant line) and the *capsicum* AC2258 (susceptible line) [29]. It has been also studied that the BW resistance is governed by two to five genes using the progeny derived from a cross between the PM687 (resistant line) and the Yolo (susceptible line) [34]. In addition to this, researches have demonstrated that the disease severity in F$_1$ hybrids is close to or lower than the generation-means of mid-parent values [29,49], and the progeny derived from BVRC 1 (resistant line) and BVRC25 (susceptible line) [36] as well as from MC4 (resistant line) and Subicho (susceptible line) exhibited the association of more than two resistance genes against *R. solanacearum*, indicating that the BW resistance would be involved in a partial dominance effect [71]. Although the complex mechanism still needs to be elucidated, the contradictory findings might result from diverse factors, including the different pepper sources of breeding lines, the different inoculation methods, the bacterial isolates, the different criteria using DI calculation, and the different environmental growth factors. It is, therefore, of our interest to further study the complex action of genetic inheritance in the pepper source used for the experiment with the comparison of other BW-resistant lines.

*4.3. Detection of Major QTL Controlling Resistance to R. solanacearum*

Multiple management strategies have been actively developed and applied to control the BW disease. However, the effects have been limited and insufficient for the control of destructive BW disease owing to the different pepper sources, the bacterial isolates, and different inoculation methods as mentioned above [26–28,63]. Besides, high disease-resistance phenotype is not always associated with a good performance of horticultural traits, such as good fruit shape, fruit size, fruit yield, and fruit quality [71]. Thus, it is crucial to understand the genetic basis for resistance to BW disease to utilize pepper breeding programs using MAS, thereby ultimately integrating BW resistance with desirable traits during a breeding process [47,48,71]. In previous studies, the pepper accessions of LS2341 and BVRC1 have been determined with a *Bw1* QTL and a major *qRRs-10.1* QTL underlying on chromosome 08 and 10, respectively [35,36]. Initially, Mimura et al. (2009) reported that the CAMS451 marker of *Bw1* QTL in linkage group 11 (LG11) was located on chromosome 01 with the comparison of a LG01 on chromosome 01 of SNU3 map, which was integrated by a genetic linkage map of an interspecific cross between *C. annuum* and *C. chinense* [35]. However, the group reported the linkage group was shifted to chromosome 08 from 01 again [72]. The current studies suggest that the LG 11 is possessed by chromosome 08 rather than 01 in *C. annuum*. Mathew (2020) recently reported that the pepper CAMS451 marker of *Bw1 QTL* lies on chromosome 08 (position: 122704651-124710667) and has annotated 44 defense-associated genes from 1 Mb upstream and downstream of the marker [73]. In addition to this, another research on the BW resistance against *R. solanacearum* demonstrated that a major *qRRs-10.1* QTL region is located on chromosome 10 (position: 56910000-69110000, 111090000-183670000) in *C. annuum*. Interestingly, 54 genes were annotated, and five putative R genes lie on the regions of 193.4–196.3 Mb, which are nearly closed to the markers of ID10-194305124 and ID10-196208712 [36]. In contrast, in the present study, we identified the major *pBWR-1* QTL region on chromosome 01 via a CIM method using the GBS analysis on the basis of the threshold levels (Figure 3), which exhibited the remarkable LODs from 5.69 to 7.06 in LG_01-1 (Table 3). Importantly, our finding shows that the

major *pBWR-1* QTL region underlies on chromosome 01 (position: 47793907–61496208, 130789593–161127926) with 12 markers, which encode 31 candidate genes in the region (Tables 3 and 4). These discrepancies of previous and our current result on the genome loci would result from a variety of materials and methods as aforementioned reasons.

*4.4. pBWR-1 Candidate Genes for Resistance to R. solanacearum*

We annotated the 31 candidate genes on the basis of sequence alignment using CM334 reference genome, KEGG, Swiss-Prot, GO, and NR databases (Table 4). Among them, four candidate genes were annotated as defense-associated genes: one gene, CA01g18650 (putative disease-resistance protein RPM1), in ch01_156505108 marker and three genes, such as nearly tandem-arrayed genes CA01g20110 (GO: 0006952, Thaumatin-like protein), CA01g20130 (putative disease-resistance protein RPM1, KO: 13457), and CA01g20140 (NB-ARC domain-containing protein), in ch01_161127926 marker. In comparison with previous publications, the identified candidate genes might not be similar to Mathew's (2020) results on chromosome 08, whereas the genes encoding LRR proteins and NB-ARC domain-containing protein on chromosome 10 in Du et al. (2019) are shared with our results, implying that these genes are possibly indispensable for BW resistance.

Previous studies have reported that pathogen defense-associated R genes are tandemly located in chromosomes [74–76]. For example, eight genes encoding an amino terminal coiled-coil domain (CC), a central nucleotide binding (NB) site, leucine-rich repeat (LRR) domain are tandemly arrayed in the *Pvr4* locus of CM334 genome in *C. annuum*. Fourteen genes encoding NB-LRR tandemly lie on the *Tsw* locus of PI159236 genome in *C. chinense*. Moreover, among of three tandem-arrayed R genes within the *qRRS-10.1* QTL, two genes, including CA10g13010 and CA10g13020, were annotated as *Bs2*, which is classified into the NB-LRR family, suggesting that NB-LRR proteins play a crucial role in disease resistance against pathogens [75]. It has been studied that elongation factor tu receptor (EFR) from *Arabidopsis* and *Bs2* from pepper are expressed in tomato plant for controlling BW and bacterial spot (BS), respectively [77]. Intriguingly, the EFR was determined as a critical component in plant defense of PAMP-triggered immunity (PTI) via the interaction between conserved PAMPs and bacterial pathogens [78]. Furthermore, Du et al. (2019) reported that CA10g12520 within the *qRRS-10.1* QTL encodes PR-1 gene, indicating the participation in the interaction of plant and pathogen via PTI. In similar line with previous results, we identified CA01g18650 and CA01g20130 encoding putative disease-resistance protein RPM1. The bacterial resistance to *Pseudomonas syringae pv. maculicola 1* (RPM1) encodes a CC-NB-LRR family protein, which is a peripheral plasma membrane protein [79,80]. The RPM1 recognizes the effector proteins of *avrB* or *avrRpm1* of *Pseudomonas syringae* in *Arabidopsis*, resulting in the rapid generation of a hypersensitive response (HR) [79–81]. Moreover, identified CA01g20140 harbors NB-ARC domain-containing protein. It has been shown that effector-triggered immunity (ETI) is associated with nucleotide-binding leucine-rich repeat (NLR) proteins [82]. A variety of plant NLRs possess an NB-ARC domain (nucleotide-binding adaptor shared by Apaf-1, R proteins, and CED4), which is able to interact with NLR domain-containing proteins, indicating that an NB-ARC domain is important for pathogen defense [83,84]. Importantly, we also identified CA01g20110 encoding Thaumatin-like protein (GO: 0006952). Previous researches have reported that Thaumatin-like proteins (TLPs) are classified into PR (pathogenesis-related protein)-5 class protein, and TLP genes are upregulated in peanut (*Arachis hypogaea* L) with the treatment of the leaf spot pathogen, *Phaeoisariopsis personata* [85], as well as in wheat (*Triticum aestivum*) by leaf rust fungus, *Puccinia triticina* [86]. In addition, constitutive expression of *Arabidopsis* Thaumatin-like protein 1 (ATLP1) in potato are observed with reduced lesions and percent reductions in response to *Alternaria solani* and *Phytophthora infestans* [87], implying that TLPs are essential in diverse biotic response. Although we cannot completely rule out the effect of differential expression of other candidate genes in the list (Table 4) and minor QTL effects that were not detected on the BW resistance in the current study, it is our endeavor for future research to focus on the understanding of genetic mechanisms, such as

inheritance factors, as well as on functional analysis of the identified candidate genes in the resistance to *R. solanacearum*.

## 5. Conclusions

In the present study, the disease symptoms and resistance for BW were evaluated by the inoculation with *R. solanacearum* using 94 $F_2$-segregating populations. Using GBS, 628,437 SNPs were identified, and the filtered 1550 SNP were subsequently constructed into the genetic linkage map displaying 16 LG. QTL analysis revealed that *pBWR-1* QTL was located on chromosome 01, and the number of 31 candidate genes were identified in the significant QTL regions. Importantly, the identified four genes encode defense resistance-associated proteins, such as CC-NB-LRR family protein, NB-ARC domain-containing protein, and Thaumatin-like proteins. Our finding will contribute to deep insights into the information for developing SNP markers associated with BW-resistant QTL as well as for developing BW-resistant cultivars in pepper breeding programs.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/horticulturae8020115/s1, Supplementary Figure S1: Distribution of the disease index after inoculation with *Ralstonia solanacearum*; Table S1: Genotyping-by-sequencing (GBS) statistics for 96 samples; Table S2: Single-nucleotide polymorphism (SNP) filtering criteria.

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