

Article

Identification of QTLs and Candidate Genes for Red Crown Rot Resistance in Two Recombinant Inbred Line Populations of Soybean [*Glycine max* (L.) Merr.]

Augustine Antwi-Boasiako ^{1,2} , Chunting Zhang ¹, Aisha Almakas ¹ , Jiale Liu ¹, Shihao Jia ¹, Na Guo ¹, Changjun Chen ³, Tuanjie Zhao ^{1,*}  and Jianying Feng ^{1,*} 

- ¹ Key Laboratory of Biology and Genetics Improvement of Soybean, Ministry of Agriculture, Zhongshan Biological Breeding Laboratory (ZSBBL), National Innovation Platform for Soybean Breeding and Industry-Education Integration, State Key Laboratory of Crop Genetics & Germplasm Enhancement and Utilization, College of Agriculture, Nanjing Agricultural University, Nanjing 210095, China; bbbantwi@yahoo.com (A.A.-B.); 2018801229@njau.edu.cn (C.Z.); aisha90@126.com (A.A.); ljl15256082024@163.com (J.L.); chulifeng@163.com (S.J.); guona@njau.edu.cn (N.G.)
- ² Council for Scientific and Industrial Research-Crops Research Institute (CSIR-CRI), Fumesua, Kumasi P.O. Box 3785, Ghana
- ³ College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China; changjun-chen@njau.edu.cn
- * Correspondence: tjzhao@njau.edu.cn (T.Z.); fengjianying@njau.edu.cn (J.F.)

Abstract: With the rapid emergence and distribution of red crown rot (RCR) across countries, durable sources of resistance against *Calonectria ilicicola* in soybean [*Glycine max* (L.) Merrill] is required to control the disease. We employed two RIL populations for the experiment. We identified 15 and 14 QTLs associated with RCR resistance in ZM6 and MN populations, respectively, totaling 29 QTLs. Six and eight QTLs had phenotypic variation above 10% in ZM6 and MN populations, respectively. We identified six (6) “QTL hotspots” for resistance to RCR from the ZM6 and MN RIL populations on chromosomes 1, 7, 10, 11, 13, and 18. Gene annotations, gene ontology enhancement, and RNA sequencing assessment detected 23 genes located within six “QTL Hotspots” as potential candidate genes that could govern RCR resistance in soybeans. Our data will generally assist breeders in rapidly and effectively incorporating RCR resistance into high-yielding accession through marker-assisted selection.

Keywords: soybean; red crown rot; resistance; candidate gene; QTL mapping



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1. Introduction

Soybean [*Glycine max* (L.) Merr.] is a significant legume crop globally because of its high nutritional values [1]. In China, soybeans are essential legumes for vegetable oil and animal feed are concerned, ranking fourth in its production globally in 2022 (FAO-STAT, <https://www.fao.org/faostat>, accessed on 31 December 2023). Economic analysis of soybean production shows a high return contributing to the economic and social impact, especially on smallholder farmers [2,3]. Hence, some researchers suggest the need to establish interventions to protect China’s economic, environmental, and sustainable practices for soybean production [4].

Unfortunately, soybean yield parameters are usually confronted with a number of stresses ranging from biotic (pests, diseases, and weeds) to abiotic (drought, salinity, and heavy metals), regulating the quantity and quality of soybean seeds and their nutritional components [5,6]. One of the key constraints impeding soybean production arises from fungal pathogens, which cause 26 different diseases [7]. Particularly in China, *Calonectria ilicicola* Boedijn and Reitsma are among the economically important pathogens as they infect several plants such as soybean, blueberry, stout camphor tree, groundnuts, alfalfa, sassafras,

and ginger [8–10]. Now, soybean red crown rot (RCR) disease caused by *C. ilicicola* is a threat to major soybean regions, especially in southern Asia. Soybean RCR negatively impacts the quantity of seed in a pod, its seed weight, and quality [11–13], thus limiting yield by 25–30% [14–16], and it is projected to reach 50–100% disease incidence on susceptible cultivars [11,14,17]. Morphologically, strains of *C. ilicicola* are characterized by anamorphic structures (macroconidia, vesicles, conidiophores, and conidia) and teleomorphic structures (perithecia, asci, and ascospores) [18,19]. *C. ilicicola* has also been identified via internal transcribed spacer (ITS) regions, calmodulin, histone3, and translation elongation factor 1- α [19,20]. Recently, novel *Calonectria* species and new hosts have been recorded in Southern China [21]. The fungus *C. ilicicola* infects the roots of soybeans, resulting in root rot advancing to pencil-like roots, damping off of younger seedlings, and untimely defoliation [22–24]. Currently, neither an effective fungicide nor a resistant cultivar controls RCR; however, agronomic practices are adopted to manage the disease [15,25,26]. The generation of RCR-resistant varieties continues as the foundation for disease management.

Research on soybean diseases is of growing interest owing to its consequences. Hence, several researchers have investigated the inherent features of disease traits via quantitative trait loci (QTL) through linkage analysis and quantitative trait nucleotides (QTNs) via genome-wide association studies (GWAS) [27,28]. The disease traits in soybeans are associated with the growing season, isolates and their diversity, and the soybean cultivar [7,29]. Thus, disease traits are complex and are controlled by several genes [30,31]. Therefore, it is crucial to identify key genes that could provide resistance to soybean crops. Molecular breeding provides an inclusive comprehension of the genetic basis underlying disease traits. Previous researchers have identified QTLs/QTNs utilizing either GWAS or linkage mapping analysis to unravel the genetic basis of soybean disease traits [31,32]. The success of QTL/QTN identification largely depends on the mapping strategies used. However, few soybean germplasm accessions show RCR resistance [25]. Hence, the application of GWAS becomes comparatively difficult as rare variants could be omitted due to the minor allele frequency (MAF) cut-off, leading to skewed phenotypic distribution toward the susceptibility genotypes [33]. On the contrary, biparental population RCR-resistance sources could be studied by utilizing the genetic background of the resistant parent. Linkage mapping analysis has proven fruitful in detecting markers linked with various soybean disease traits. For instance, linkage mapping has successfully been applied in soybean diseases such as rust [34,35], bacterial leaf pustule [32,36], mold white [37,38], *Phytophthora* root rot [39–41], and frog eye leafspot [42,43], resulting in detecting QTLs. Conversely, bi-parental mapping populations have yet to be employed in the search for QTL related to RCR resistance in soybeans. Therefore, the current investigation used a high-density linkage map of two RIL populations, namely ZM6 and MN, and evaluated two different times in an environment to identify QTLs and mine for potential candidate genes for RCR resistance in soybeans. We sought to identify QTLs and potential genes governing regulating RCR resistance in soybeans. This study deepens our understanding of the genetics of RCR resistance in soybeans, thus contributing to the identification, development, and application of markers related to RCR resistance for marker-assisted selection toward developing resistant cultivars.

2. Materials and Methods

2.1. Plant Material

After preliminary RCR resistance evaluation of parents of six recombinant inbred line (RIL) populations (LM6, ZM6, M6T, M6T, MN, ZN), the parents of ZM6 and MN RIL populations showed significant resistance differences, so these two populations were selected for mapping RCR resistance QTL. Both RIL populations were developed through the single-seed descent (SSD) method by advancing to the seventh generation. The ZM6, consisting of 122 lines, was developed by crossing a disease-sensitive variety, Zhengyang, and a disease-tolerant cultivar, Meng8206 to *C. ilicicola*. Also, MN, consisting of 98 lines, was developed using the soybean cvs. M8180 and NN1138-2, which are resistant and susceptible to *C. ilicicola* strain Y62, respectively. All the RIL population accessions were sourced

from the Soybean Germplasm Gene Bank, located at the National Centre for Soybean Improvement (Ministry of Agriculture), Nanjing Agricultural University, Nanjing, China.

2.2. Pathogen Culture and Inoculation

The *C. ilicicola* strain Y62 was provided by the College of Plant Protection, Nanjing Agricultural University, Nanjing, China. The fungi were maintained on V8 media plates (90 mm) at 26 °C for short-term use by subculturing. Pathogen inoculation was carried out following the protocol of Jiang et al. (2020) [25].

2.3. Growth Conditions, Experimental Conditions, and Design

The planting media consisted of a mixture of vermiculite and nutritive soil in equal volumes (1:1, *v/v*). The media was autoclaved. After cooling at ambient temperature for two days, the media was combined with an inoculum–soil mixture to achieve a concentration of 2% (*w/v*) and then filled into the plastic pot as the growing media as described by Jiang et al. (2020) [25]. The seeds for planting consist of the two RIL populations, along with their parents. Each pot was planted with ten seeds, and the surface was then covered with a two-millimeter layer of the growing medium. Pots were positioned within a container, and water was directed to the pots through their drainage openings to make sure they were completely saturated in a greenhouse maintained at a temperature of 26 °C and a relative humidity of 50%. Water was supplied to the container to maintain soil moisture throughout the assay. To mitigate against potential location-related impacts, the plastic pots in the greenhouse were changed every two days. The study used a completely randomized design (CRD) with three replications.

2.4. Phenotypic Data

Phenotypic data were taken on the emergence rate (ER), survival rate (SR), and disease severity (DS). The data on the traits were taken twice. The first data set was taken between February and April 2023, termed as the first (1st) data set. The second time data set was taken between June and August 2023 as the second (2nd) data set.

2.4.1. Assessment of the Rate of Emergence and Survival for Resistance to *Calonectria ilicicola*

The soybean accessions were scored for emergence rate on the 5th day after planting (DAP). The emergence rate was expressed as the total number of seeds that emerged out of the total number of seeds planted expressed in percentage. The survival rate was taken on the 12th DAP. Survival rate was calculated as the total number of plants alive out of the total number of plants that emerged expressed in percentage.

2.4.2. RIL Populations Lines Response to *Calonectria ilicicola* Infection

The seedlings were evaluated for *C. ilicicola* resistance on the 14 DAP according to Jiang et al. (2020) [25]. The seedlings were gently removed from the pot, and the roots were washed to ensure that the roots were free from soil for visual examination. The resistance level of each RIL was determined using DS in addition to their SR and ER traits. A threshold of $SR > 0.90$ and $ER > 0.85$ was used as a standard to identify accessions with resistance. The SR helped to determine the mortality rate of the seedling, which was incorporated into the disease resistance rating scale. The DS employs a numerical scale ranging from 0 to 5, as indicated in Table 1. These were employed to categorize RILs according to their responses to *C. ilicicola* infection. The average of the three repeats of each RIL was further used to classify them using $y \leq 1.5$, $1.5 \leq y \leq 2.5$, $2.5 \leq y \leq 3.5$, $3.5 \leq y \leq 4.5$, and $y > 4.5$ as highly resistant, resistant, moderately susceptible, susceptible, and highly susceptible to *C. ilicicola*, respectively.

Table 1. Classification for the disease severity of red crown rot.

Scale	Damage Degree	Resistance Degree
0	No visible sign of necrotic lesions on the root	Immune
1	Only the tap root exhibits small necrotic lesions without obvious changes in its form and shape	Highly Resistant
2	Necrotic lesions spread to the crown and root system and seedling mortality less than 10%	Resistant
3	Roots show serious necrotic lesions with less than 50% loss by rot and seedling mortality of 10–20%	Moderately Susceptible
4	Roots show serious necrotic lesions with more than 50% root loss by rot and seedling mortality of 21–50%	Susceptible
5	Over 50% of root loss by rot with seedling mortality of more than 50%	Highly Susceptible

2.4.3. Analysis of Phenotypic Data

The GenStat software, version 12 (VSN International Ltd., Hemel Hempstead, UK), was utilized to generate descriptive statistics for the ER, SR, and DS traits in both RIL populations as well as their parents. These statistics included the mean, range, degree of variation (CV, %), skewness, and kurtosis. A Pearson correlational study was conducted to examine the relationship between ER, SR, and DS. The results were shown using the Corrplot package in R (version 0.84, Auckland, New Zealand). The level of significance was set at $p < 0.05$ [44].

2.5. SNP Genotyping and Linkage Map Construction for ZM6 and MN RIL Populations

We extracted the genomic DNA using young and fresh leaves of the entire populations of ZM6 and MN RILs in addition to the parents, following the standard established by [45]. The DNA library construction, sequencing, SNP acquisition, and integration of Bin/SLAF (specific-locus amplified fragment) markers were performed for the ZM6 and MN populations. For the ZM6 population, bin markers were used for genotyping [46,47]. Also, the MN population was genotyped using SLAF markers [46,47]. The high genetic maps of the ZM6 and MN populations comprised 2601 bin markers and 2062 SLAF markers, respectively. The combined length of the ZM6 and MN maps was 2630.22 cM and 2054.5 cM, having a mean distance of 1.02 cM and 1.36 cM between neighboring markers, respectively (Table S1). The mean length of each linkage group was 131.51 cM for the ZM6 linkage map and 102.73 cM for the MN linkage map. The average number of markers per chromosome was 130.05 and 103.1 from the ZM6 and MN populations, respectively (Table S1).

2.6. QTL Mapping for Emergence Rate, Survival Rate, and Disease Severity

The WinQTLCart (version 2.5, Raleigh, NC, USA) application was used to locate QTLs via composite interval mapping (CIM) [48]. The window size, working speed, and control were configured to be 10 cM, 1 cM, and less than 5 cM, respectively [49]. The LOD thresholds for identifying significant QTLs were established at 2.5, using 1000 permutations for each test and a type I error rate of 5% [50]. The precise physical positions of individual QTLs on all chromosomes were determined using the MapChart (version 2.1, Wageningen, Netherlands) tool [51]. Names were assigned to the QTLs by adhering to the accepted nomenclature system [52]. For instance, for the QTL denoted as *qER-7-1zm6*, the letter *q* stands for QTL preceded every QTL name, which was followed by a two-letter descriptor of the ER trait, 7 refers to the specific chromosome on which the QTL was identified, 1 displays the number of each QTL trait on the chromosome, and *zm6* represents the ZM6-RIL population where the QTL existed.

2.7. Mining of Candidate Genes for Major QTLs

We obtained the complete genomic data from the main “QTL hotspots” by utilizing the internet-based resource libraries from Phytozome (<http://phytozome.jgi.doe.gov> (accessed on 13 January 2024)) and SoyBase (<http://www.soybase.org> (accessed on 13 January 2024)), which also assisted in candidate gene identification along with the data obtained from previously published publications. We used the Gene Ontology (GO) online platform to

analyze the identified potential genes. GO enrichment analysis was performed for all the QTL hotspots using agriGo (version 2.0, Beijing, China) [53].

The available RNA sequencing dataset by Kobayashi et al. (2022) [54] and our unpublished data were applied to investigate the transcription of identified candidate genes in different soybean tissues and phases of development. A heat map was generated using TBtools version 1.6 to visually represent the changes in gene expression trends for the anticipated candidate genes [55].

3. Results

3.1. Variability Characteristics of the Mapping Traits for the RIL Populations

The use of parents with varied phenotypes is necessary for the creation of mapping populations, and this is crucial for maximizing the effectiveness of QTL discovery [56]. For this investigation, two mapping RIL populations were utilized: ZM6 RIL, which originated from the cross between Zhengyang (♀) and Meng8206 (♂), and MN, which originated from the cross between M8180 (♀) and NN1138-2 (♂) (Table 2). The ZM6 and MN RIL populations display the mean, range (minimum and maximum value), standard deviation, skewness, and kurtosis of the ER, SR, and DS for the two RIL populations (ZM6 and MN) and their parents (Table 2). These measurements were examined for two conservative times (first and second). The parental genotypes exhibited relatively large variations for ER, SR, and DS (Table 2). The RILs mostly showed a transgressive segregation. The skewness and kurtosis were mostly less than 1, which is typical for quantitative attributes, implying that the populations are suitable for conducting QTL mapping (Table 2). Furthermore, the measured variables (ER, SR, and DS) exhibited a distinct range of distribution across both RIL populations (Figure 1). Based on the three traits screened, there were significant positive correlation coefficients between SR and ER in both mapping populations, while significant negative correlation coefficients existed between DS against ER and SR (Figure 2).

Table 2. Response of two soybean RIL populations and their parents to RCR.

Population/ Environment ^a	Trait ^b	Mean of Parent (%) ^c		RIL Populations ^d				
		♀	♂	Mean ± SE	Min.	Max.	Kurtosis	Skewness
ZM6/2	ER	73.33 ± 3.33	83.33 ± 3.33	83.11 ± 0.00	63.33	100.00	−0.86	0.13
	SR	82.14 ± 3.57	100 ± 0.00	86.07 ± 0.00	54.76	100.00	−0.09	−0.63
	DS	3.33 ± 0.33	1.00 ± 0.00	2.83 ± 1.00	1.00	4.00	−0.50	−0.10
ZM6/1	ER	76.67 ± 3.33	93.33 ± 3.33	80.44 ± 1.00	56.67	100.00	−0.30	−0.32
	SR	82.74 ± 3.90	100 ± 0.00	81.79 ± 1.00	39.68	100.00	−0.05	−0.59
	DS	4.33 ± 0.33	2.00 ± 0.00	3.46 ± 1.00	1.00	4.33	−0.50	−0.10
MN/2	ER	100.00 ± 0.00	80.00 ± 0.00	82.50 ± 5.20	35.67	100.00	1.31	−1.08
	SR	100.00 ± 0.00	83.33 ± 6.93	96.45 ± 0.04	74.17	100.00	3.86	−1.93
	DS	1.67 ± 34.64	3.67 ± 5.75	2.46 ± 6.09	1.00	4.00	−0.66	0.30
MN/1	ER	96.67 ± 5.97	70.00 ± 0.00	78.44 ± 3.92	60.00	90.00	3.67	−1.36
	SR	100.00 ± 0.00	76.67 ± 8.00	87.86 ± 3.00	52.86	100.00	1.28	−1.03
	DS	2.00 ± 0.00	3.6 ± 6.00	3.00 ± 6.00	1.33	4.00	−0.53	−0.52

^a Environment: the first and second time screenings (1 and 2) in each RIL population. ^b ER—emergence rate, SR—survival rate, and DS—disease severity. ^c Zhengyang (♀) and Meng8206 (♂) for ZM6 RILs, while M8180 (♀) and NN1138-2 (♂) for MN RILs. ^d SE—standard error of mean, min—minimum, and max—maximum.

3.2. QTL Detected in the ZM6 Population for Red Crown Rot Resistance in Soybean

The composite interval mapping (CIM) strategy was employed using the 2601 bin markers obtained among the ZM6 RIL population and was polymorphic between the parents. A total of 15 QTL for the three traits in ZM6 RIL population were located on eight different chromosomes (Chr06, Chr07, Chr08, Chr10, Chr11, Chr13, Chr17, and Chr18), of which 5, 6, and 4 were for ER, SR, and DS, respectively, and each QTL had phenotypic variance explained (PVE) ranging between 8.40 and 14.87% (Table 3; Figure 3). The 5 QTL for ER consisted of *qER-7-1zm6* (Chr07), *qER-8-1zm6* (Chr08), *qER-10-1zm6* and *qER-10-2zm6* (Chr10), and (*qER-11-1zm6*) (Chr11) with their PVE ranging from 8.40 to 10.70%. With the

exception of the 2 QTL on Chr10 (*qER-10-1zm6* and *qER-10-2zm6*), which inherited its alleles from the male parent, Zhengyang, the remaining three QTLs (*qER-7-1zm6*, *qER-8-1zm6*, and *qER-11-1zm6*) had their alleles from the female parent, Meng8206 (Table 3).

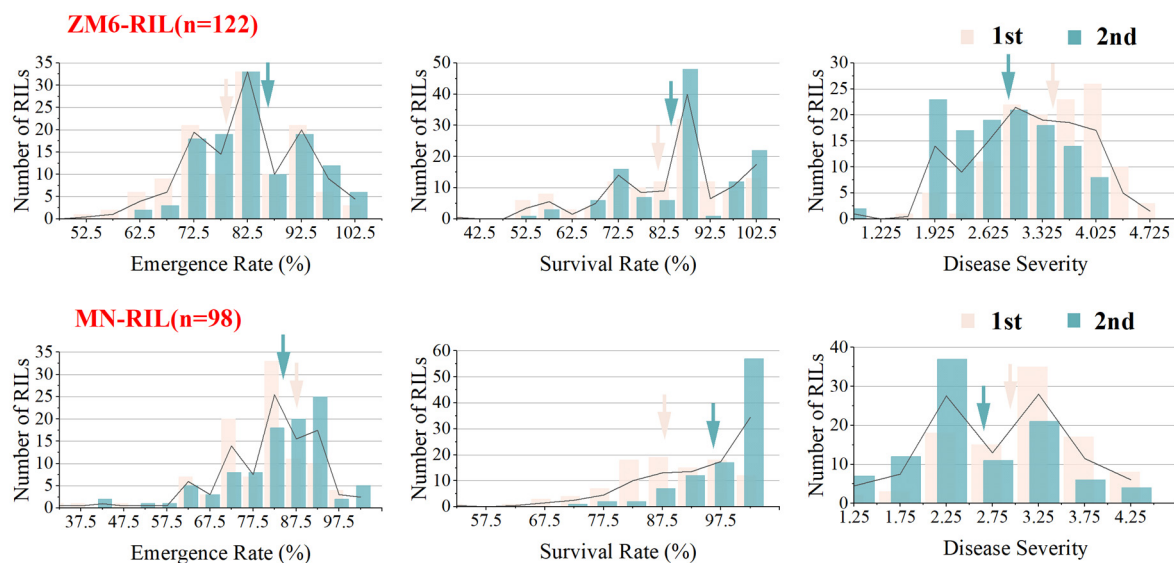


Figure 1. Frequency distribution of emergence rate, survival rate, and disease severity in ZM6 and MN RIL populations. The phenotype was averaged from the first and second time screenings in each RIL population. The trend shows the moving average. The arrows show the mean value of corresponding. The horizontal and vertical axis represent trait value and number of genotypes, respectively.



Figure 2. Pearson correlation matrix among the emergence rate (ER), survival rate (SR), and disease severity (DS) used to screen for red crown rot resistance in ZM6 and MN RIL populations (ZM6, upper part, and MN, lower part of the matrix). The phenotype was averaged from the first and second time screenings of the study in each RIL population. The asterisk shows significance at $p < 0.05$. The color scale of each correlation coefficient is shown in the color legend on the right-hand side of the correlation matrix.

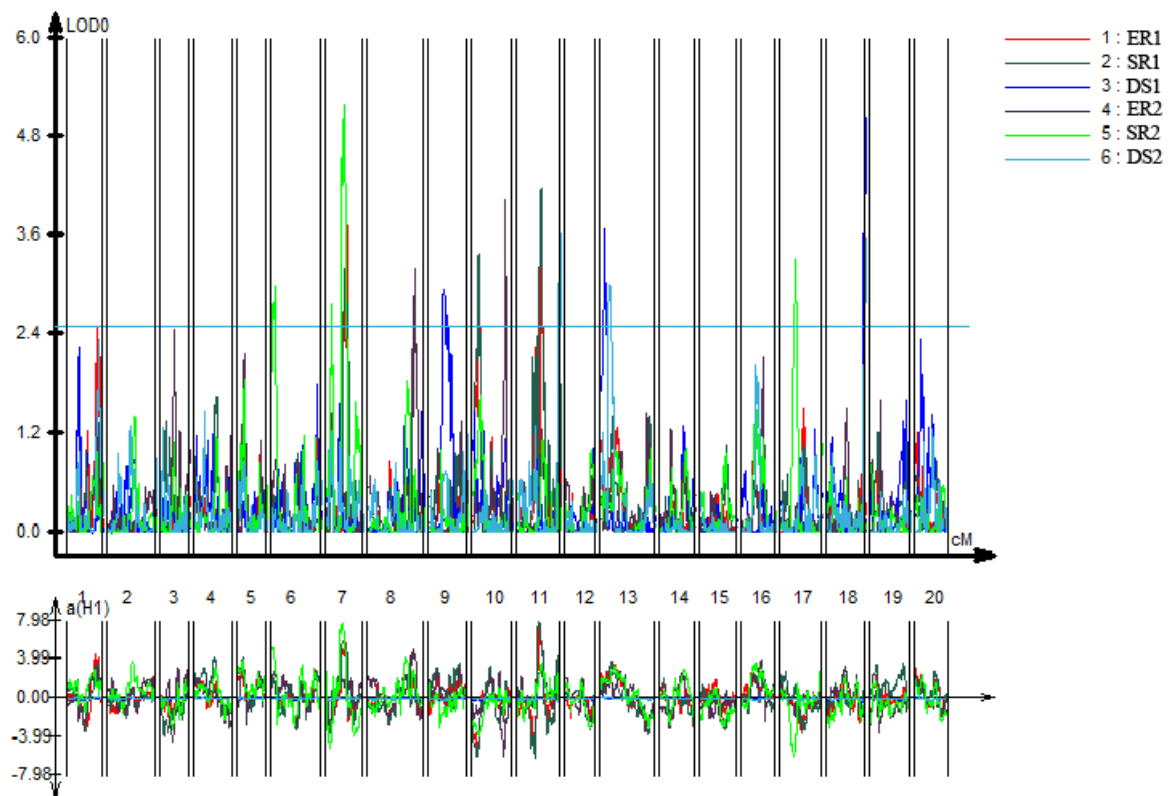


Figure 3. Genome-wide view of QTL detected in the ZM6 RIL population for soybean root crown rot disease via the CIM strategy. The phenotypic data were obtained from two time screenings (first time and second time). The disease resistance was screened by three traits: emergence rate (ER), survival rate (SR), and disease severity (DS). The y-axis of the top figure represents a logarithm of odd value with a threshold of 2.5 (sea blue line), while the x-axis represents the chromosome (Chr) number in the soybean genome. The bottom figure denotes the additivity of alleles and their origin from either of the parents: positive values represent allele from Meng8206 (♀), while negative values emanate from Zhengyang (♂). The color legend is shown on the right-hand side of the top figure.

Table 3. QTLs detected for RCR disease resistance via ER, SR, and DS in the ZM6 RIL population via the CIM strategy.

QTL ^a	Pos (cM) ^b	LOD ^c	Add ^d	PVE (%) ^e	CI (cM) ^f	Physical Region (bp)	Env ^g
<i>qER-7-1zm6</i>	64.51	3.82	5.81	10.37	61.4–69.7	15,375,767–17,747,973	1
<i>qER-8-1zm6</i>	150.81	3.22	5.04	8.40	147.7–154.7	41,485,100–42,915,255	2
<i>qER-10-1zm6</i>	16.61	3.25	−5.24	8.56	12.8–19.6	1,694,367–2,679,837	1
<i>qER-10-2zm6</i>	106.41	4.04	−6.00	10.70	105.1–109.9	43,900,754–44,741,960	2
<i>qER-11-1zm6</i>	73.81	3.86	7.20	10.40	71.2–75.5	14,962,695–15,949,296	1
<i>qSR-6-1zm6</i>	9.71	2.97	5.32	7.78	6.7–15.6	1,813,130–3,196,555	2
<i>qSR-7-1zm6</i>	48.81	4.55	7.08	12.71	46.2–50.1	9,304,376–10,428,532	2
<i>qSR-7-2zm6</i>	56.01	5.19	7.71	14.87	53–62	14,134,797–15,903,280	2
	59.21	3.21	6.00	8.62	52.7–61.4	14,134,797–15,452,798	1
<i>qSR-10-1zm6</i>	16.61	3.37	−6.00	8.88	10.3–19.6	1,603,735–2,732,880	1
<i>qSR-11-1zm6</i>	76.41	4.19	7.66	11.34	75.5–81.8	15,676,274–16,816,800	1
<i>qSR-17-1zm6</i>	46.41	3.32	−5.93	8.74	32.8–53.7	6,777,393–9,645,325	2
<i>qDS-11-1zm6</i>	139.11	3.81	−0.26	9.55	136–140.8	37,603,249–38,850,696	2
<i>qDS-13-1zm6</i>	8.91	3.69	0.27	9.26	3.1–11.2	4,552,834–5,592,448	1
<i>qDS-18-1zm6</i>	120.11	3.63	−0.28	9.57	118–122.7	59,218,992–60,685,675	1
<i>qDS-18-2zm6</i>	128.51	5.04	−0.32	12.95	124.8–129.5	61,300,197–62,014,706	1
	128.51	3.57	−0.25	8.89	124.2–129.5	60,909,812–62,014,706	2

^a QTLs detected in different environments at the same, adjacent, or overlapping marker intervals were considered the same QTL. ^b Pos—Position of QTL in centiMorgan. ^c LOD—Logarithm of odds. ^d Add—Additive, indicating the origin of beneficial alleles; positive values represent allele from Meng8206 (♀), while negative values emanated from Zhengyang (♂). ^e PEV—phenotypic variance explained (%) expressed by the QTL. ^f CI—Confidence interval. ^g Env—Environment, the phenotypic data obtained from two time screenings (first time and first time).

Six QTLs were mapped for SR across five chromosomes: Chr06 (*qSR-6-1zm6*), Chr07 (*qSR-7-1zm6*, and *qSR-7-2zm6*), Chr10 (*qSR-10-1zm6*), Chr11 (*qSR-11-1zm6*), and Chr17 (*qSR-17-1zm6*) (Table 3). Out of these, *qSR-7-2zm6* had the most prominent QTL with a LOD value of 5.19, accounting for 14.87% of the phenotypic variation, which was followed by *qSR-7-1zm6* with a LOD score of 4.55 and a difference in phenotype of 12.71% (Table 3). Four QTLs influencing DS were detected across three chromosomes (Chr11, Chr13, and Chr18), accounting for an average of 10.04% of PVE (Table 3). The Chr011 and Chr13 harbored one QTL each (*qDS-11-1zm6* and *qDS-13-1zm6*, respectively), while Chr18 had two QTL (*qDS-12-1zm6* and *qDS-12-2zm6*). Figure 4 shows the location of QTLs on the linkage genetic map of the ZM6 RIL population.

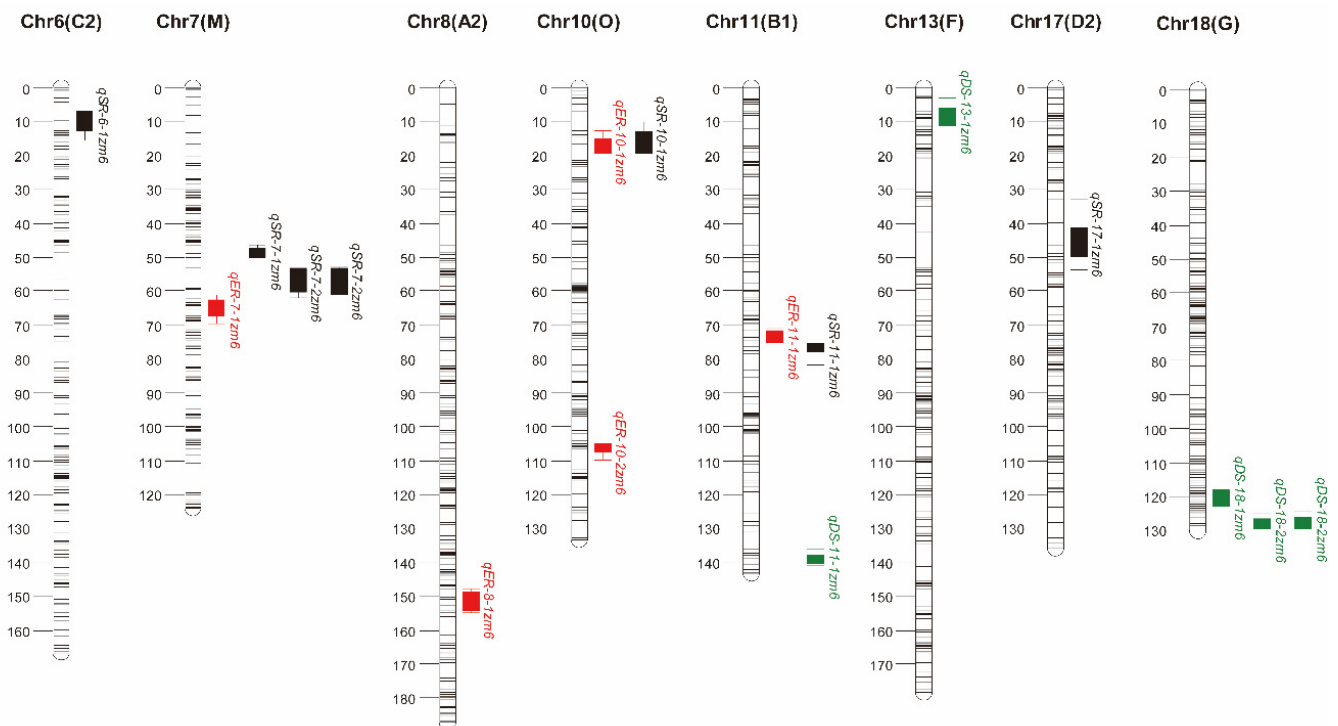


Figure 4. Location of QTLs on the genetic linkage map of ZM6 RIL population. Distances among markers are indicated using the physical location to the left of the populations and the QTL names located at their position on the right side. Chr=Chromosome. Colored bars represent different QTLs.

3.3. QTL Detected in MN Population for Red Crown Rot Resistance in Soybean

The MN RIL population detected 14 QTLs across eight chromosomes (Chr01, Chr02, Chr04, Chr08, Chr10, Chr13, Chr15, and Chr17) mapped for ER, SR, and DS comprising 5, 4, and 5 QTLs, respectively, with their phenotypic variation ranging from 8.69% to 25.24% (Figure 5; Table 4). For the ER, the five QTLs, namely, *qER-1-1mn*, *qER-10-1mn*, *qER-8-1mn*, *qER-8-2mn*, and *qER-15-1mn* had their LOD values in the range of 2.63 to 7.61 (Table 4). The QTLs for the SR were located on Chr01 (*qSR-1-1mn*, and *qSR-1-2mn*), Chr02 (*qSR-2-1mn*), and Chr17 (*qSR-17-1mn*) and their PVE ranged from 8.68% to 10.7% and their alleles were inherited from NN1138-2 (σ) except *qSR-2-1mn* (Table 4). Five QTLs, namely, *qDS-1-1mn*, *qDS-1-2mn*, *qDS-1-3mn*, *qDS-4-1mn*, and *qDS-13-1mn*, were mapped for DS with PVE ranging from 10.51% to 12.28% (Table 4). Two QTLs (*qER-1-1mn* and *qSR-1-2mn*) were detected on Chr01 across the second environment, accounting for 33.93% of the phenotypic variance, suggesting it is relative stability (Table 4). These two QTLs colocalized within the genomic interval 37,147,226–38,075,381 bp. Figure 6 shows the location of QTLs on the genetic linkage map of the MN RIL population.

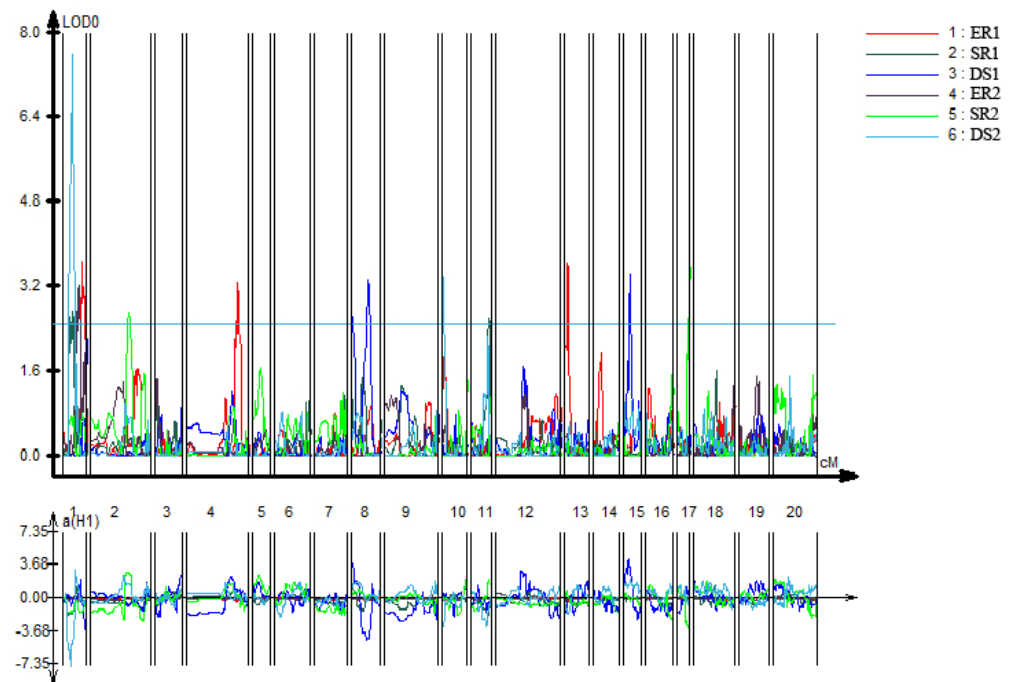


Figure 5. Genome-wide view of QTL detected in MN RIL population for root crown rot resistance via the CIM strategy. The phenotypic data were obtained from two time screenings (first time and second time). The disease resistance was screened by three traits: emergence rate (ER), survival rate (SR), and disease severity (DS). The y-axis of the top figure represents a logarithm of odd value with a threshold of 2.5 (sea blue line), while the x-axis represents chromosome (Chr) number in the soybean genome. The bottom figure denotes the additivity of alleles and their origin from either of the parents: positive values represent alleles from M8180 (♀), while negative values emanate from NN1138-2 (♂). The color legend is shown on the right-hand side of the top figure.

Table 4. QTLs detected for RCR disease resistance via ER, SR, and DS in the MN RIL population via the CIM strategy.

QTL ^a	Pos(cM) ^b	LOD ^c	Add ^d	PVE (%) ^e	CI (cM) ^f	Physical Region (bp)	Env ^g
<i>qER-1-1mn</i>	27.11	7.61	−7.24	25.24	25–29	8,823,531–22,021,358	1
<i>qER-10-1mn</i>	0.01	3.38	−3.81	10.72	0–8	2,275,280–4,174,791	1
<i>qER-8-1mn</i>	0.01	2.63	3.88	9.59	0–10	14,650,727–11,805,246	2
<i>qER-8-2mn</i>	48.71	3.35	−4.51	12.92	33.2–57.9	8,218,976–18,160,078	2
<i>qER-15-1mn</i>	16.11	3.44	4.31	12.66	13.5–22.3	5,519,255–113,832,93	2
<i>qSR-2-1mn</i>	111.51	2.67	2.97	9.32	105.5–125.6	7,361,306–15,293,225	1
<i>qSR-17-1mn</i>	34.01	2.91	−3.04	9.83	31.4–34.6	18,508,753–33,427,203	1
<i>qSR-1-1mn</i>	19.81	2.64	−1.83	10.7	14.2–23.3	3,617,559–8,823,788	2
<i>qSR-1-2mn</i>	29.01	2.73	−1.68	8.69	27.1–35	21,174,218–44,479,895	2
<i>qDS-1-1mn</i>	37.91	2.74	0.25	10.6	32.8–41.3	10,404,837–43,932,907	1
<i>qDS-1-2mn</i>	46.61	3.22	0.27	11.7	44.8–52.6	38,197,263–49,324,405	1
<i>qDS-1-3mn</i>	56.71	3.67	−0.26	11.84	51.4–58.2	41,313,930–50,832,292	2
<i>qDS-4-1mn</i>	154.41	3.28	−0.25	10.51	152.4–164	11,631,171–36,956,769	2
<i>qDS-13-1mn</i>	3.41	3.65	−0.27	12.28	0–7.8	688,713–5,592,448	2

^a QTLs detected in different environments at the same, adjacent, or overlapping marker intervals were considered the same QTL. ^b Pos—Position of QTL in centiMorgan. ^c LOD—Logarithm of odds. ^d Add—Additive, indicating the origin of beneficial alleles; positive values represent alleles from Meng8206 (♀), while negative values emanate from Zhengyang (♂). ^e phenotypic variance explained (%) expressed by the QTL. ^f CI—Confidence interval of the QTL. ^g Env—Environment.

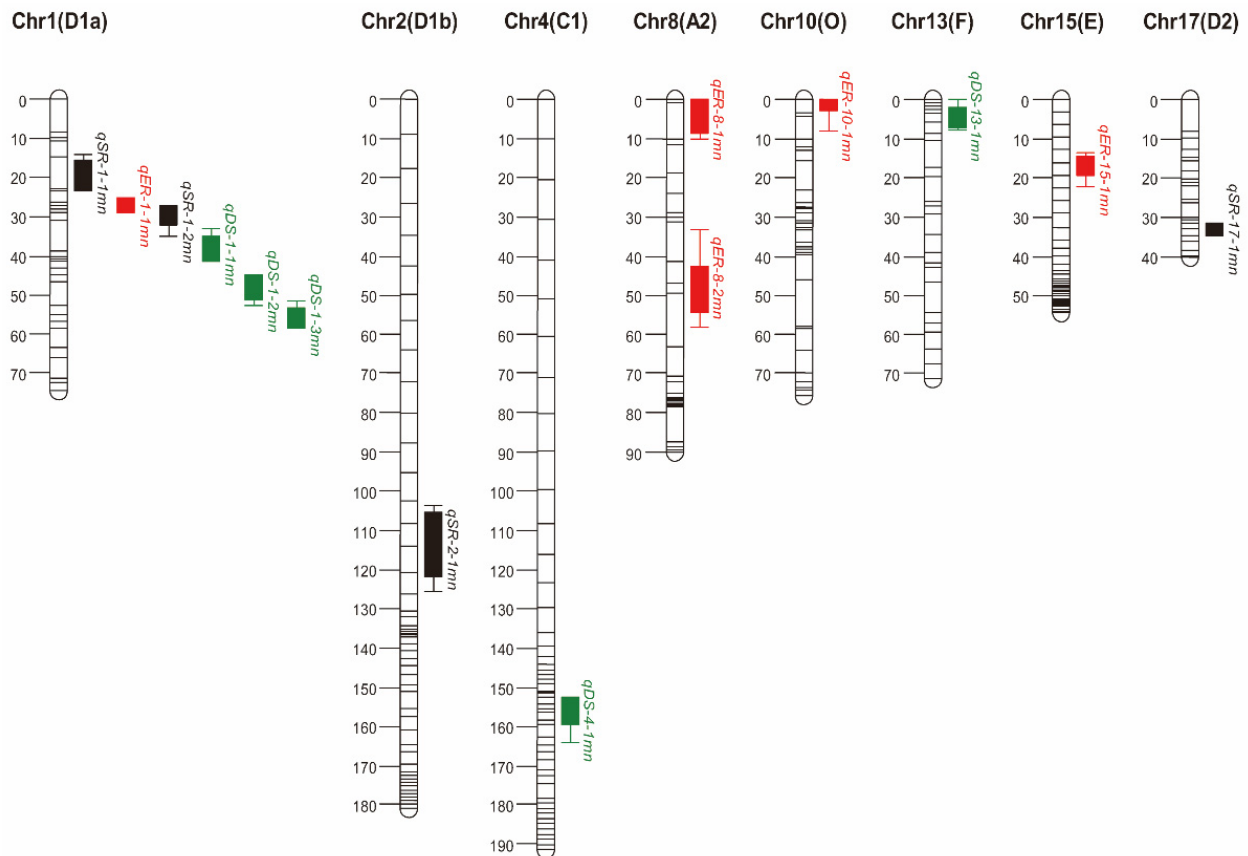


Figure 6. Location of the QTLs on the genetic linkage map of MN RIL populations. Distances among markers are indicated using the physical location to the left of the populations and the QTL names located at their position on the right side. Colored bars represent different QTLs.

3.4. Colocalization of QTLs in the QTL Hotspot

The QTL hotspot refers to the concentrated area on a chromosome that hosts many QTLs related to distinct traits. The stability of QTL across mapping populations and environments is essential for their use in practical plant breeding [57]. Hence, our stable QTLs were detected either linked to at least two of the traits (ER, SR, or DS) across an environment (first and second time of screening) within a population or across a population (found in ZM6 and MN). The QTLs *qER-1-1mn* and *qSR-1-2mn* were detected on Chr01 for two conservative times during the screening; hence, the stable QTL was termed as Hotspot A (Table 5). The QTLs were stable and are colocalized within the genomic interval of 8,823,531–44,479,895 bp. The mapping results of the ZM6 population found two pairs of QTLs, namely, *qSR-7-2zm6* colocalized within the genomic region of 14,134,797–15,903,280 bp across the two environments and renamed as QTL Hotspot B (Table 5). Also, *qER-10-1zm6* and *qSR-10-1zm6* colocalized within genomic interval 1,603,735–2,732,880 bp, termed as QTL Hotspot C. Hotspot D refers to QTL *qER-11-1zm6* in addition to *qSR-11-1zm6* colocalized within the genomic interval of 14,962,695–16,816,800. Interestingly, a stable QTL *qDS-13-1zm6* and *qDS-13-1mn* colocalized within genomic interval 68,8713–5,375,644 bp was detected across the MN and ZM6 population and named QTL Hotspot E (Tables 3–5). The QTLs *qSR-7-2zm6* and *qDs-18-2zm6* were constantly found in ZM6 in the first and second screenings, indicating that they were stable; they were thereafter classified as QTL hotspots B and F, respectively (Table 5).

Table 5. The six QTL hotspots identified in the ZM6 and MN RIL populations.

QTL Hotspot Name	QTL Name	LOD ^a	Add ^b	PVE (%) ^c	Physical Region (bp)
Hotspot A	<i>qER-1-1mn</i>	7.61	−7.24	25.24	8,823,531–44,479,895
	<i>qSR-1-2mn</i>	2.73	−1.68	8.69	
Hotspot B	<i>qSR-7-2zm6</i>	5.19	7.71	14.87	14,134,797–15,903,280
		3.20	6.00	8.62	
Hotspot C	<i>qER-10-1zm6</i>	3.25	−5.24	8.56	1,603,735–2,732,880
	<i>qSR-10-1zm6</i>	3.37	−6.00	8.88	
Hotspot D	<i>qER-11-1zm6</i>	3.86	7.20	10.40	14,962,695–16,816,800
	<i>qSR-11-1zm6</i>	4.19	7.66	11.34	
Hotspot E	<i>qDS-13-1zm6</i>	3.81	−0.26	9.55	688,713–5,592,448
	<i>qDS-13-1mn</i>	3.65	−0.27	12.28	
Hotspot F	<i>qDs-18-2zm6</i>	5.04	−0.32	12.95	60,909,812–62,014,706
		3.57	−0.25	8.89	

^a LOD—Logarithm of odds. ^b Add—Additive indicating the origin of beneficial alleles; positive values represent alleles from Meng8206 (♀), while negative values emanate from Zhengyang (♂). ^c PVE—phenotypic variance explained (%) explained by the QTL.

3.5. Candidate Gene Mining within Major “QTL Hotspots”

One of the goals of QTL mapping research is to identify possible candidate genes. This is made possible by the presence of complete genetic information and detailed gene descriptions. Therefore, model genes within the QTL hotspot and their corresponding gene annotations were obtained via SoyBase (<https://www.soybase.org>, retrieved on 26 December 2023) using soybean genome version 2. A total of 1279 gene models were found in the genomic region for each of the significant “QTL hotspots,” of which 529, 116, 118, 122, 274, and 120 genes were found within hotspots of A, B, C, D, E, and F, respectively (Table S3).

The gene ontology (GO) analysis of enrichment was conducted using the web-based toolbox agriGO (version 2.0, Beijing, China) [53]. It enabled the depiction of the main categorizations of biological process (BP), molecular function (MF), and cellular component (CC) (Figures S1–S3). We observed the highest number of genes in CC followed by BP, and MF was the least for all the QTL hotspots, as expected for QTL hotspot C, for which the BP was higher than the CC, and the MF recorded the lowest number of genes (Figures S1–S3). Analysis of the GO showed that the majority of the genes associated with the six “QTL hotspots” in CC engage in the cell, cell part, and organelle processes; the BP partake largely in processes involving cellular, metabolic, and single-organism processes; and the MF genes mainly engage in the activities of catalytic, binding, and transport (Figures S1–S3).

Using gene annotation descriptions, existing research findings, and GO enrichment examination, our study identified 32, 12, 15, 12, 25, and 15 potential genes from “QTL Hotspot A”, “QTL Hotspot B”, “QTL Hotspot C”, “QTL Hotspot D”, “QTL Hotspot E”, and “QTL Hotspot F”, totaling 111 predicted genes (Table S4). These genes play a role in controlling disease infection development, including developing resistance and innate immune response. They are involved in various processes such as signal transduction; biosynthetic processes involving salicylic acid, lignin, and flavonoid; phenylpropanoid metabolic processes; and cell death. To enhance the accuracy of the earlier-predicted list of candidate genes, we used an available online database by Kobayashi et al. (2022) [54] (Table S5) and that of our RNA-Seq data (unpublished, though is available upon request). According to the RNA-seq data, 23 genes from a total of 111 projected candidate genes exhibited a significantly greater level of transcription log₂ (FC) of ≥1 in the root, stem, R2-1d, R2-2d, S-1d, and S-2d (Figure S4A–F; Table 6). These include five genes (*Glyma.01G127100*, *Glyma.01G126600*, *Glyma.01G112300*, *Glyma.01G127200*, and *Glyma.01G127700*); two genes (*Glyma.07G133900*, and *Glyma.07G134100*); five genes (*Glyma.10G019900*, *Glyma.10G023300*, *Glyma.10G023400*, *Glyma.10G028200*, and *Glyma.10G025700*); three genes (*Glyma.11G181200*, *Glyma.11G192300*, and *Glyma.11G193600*); three genes (*Glyma.13G066100*, *Glyma.13G069200*, and *Glyma.13G076200*); and five genes (*Glyma.18G287000*, *Glyma.18G289100*, *Glyma.18G289600*,

Glyma.18G293200, and *Glyma.18G293300*) from QTL Hotspots A, B, C, D, E, and F, respectively (Figure S4; Table 6). Therefore, the 23 genes are potential candidates for regulating *C. ilicicola* resistance in soybeans. Nevertheless, additional functional validation is required to verify their specific functions in modulating resistance to soybean RCR disease.

Table 6. Predictive gene annotation information within the six QTL hotspots identified in the ZM6 and MN RIL populations.

QTL Hotspot	Gene Mapped IDs ^a	Annotation Descriptions ^b
A	<i>Glyma.01G127100</i>	Disease resistance-responsive; Dirigent-like protein
	<i>Glyma.01G126600</i>	Disease resistance-responsive; Dirigent-like protein
	<i>Glyma.01G112300</i>	Signal transduction; Leucine Rich Repeat
	<i>Glyma.01G127200</i>	Disease resistance-responsive; Dirigent-like protein
	<i>Glyma.01G127700</i>	Signal transduction; Defense response
B	<i>Glyma.07G133900</i>	Lignin catabolic process; multicopper oxidase
	<i>Glyma.07G134100</i>	Lignin catabolic process; multicopper oxidase
C	<i>Glyma.10G019900</i>	Glutathione metabolic process; glutathione S-transferase
	<i>Glyma.10G023300</i>	Protein phosphorylation; serine/threonine protein kinase
	<i>Glyma.10G023400</i>	Protein phosphorylation; serine/threonine protein kinase
	<i>Glyma.10G028200</i>	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
	<i>Glyma.10G025700</i>	F-box family protein; protein binding
D	<i>Glyma.11G181200</i>	F-box family protein; protein binding
	<i>Glyma.11G192300</i>	Oxidation-reduction process; pheophorbide an oxygenase
	<i>Glyma.11G193600</i>	Cellular glucan metabolic process; cell wall biogenesis
E	<i>Glyma.13G066100</i>	DNA repair; ATP binding
	<i>Glyma.13G069200</i>	Zinc finger (AN1-like) family protein
	<i>Glyma.13G076200</i>	Defense response; Signal transduction; Leucine Rich Repeat
F	<i>Glyma.18G287000</i>	Defense response; signal transduction
	<i>Glyma.18G289100</i>	ATP binding, hsp70 protein
	<i>Glyma.18G289600</i>	ATP binding, hsp70 protein
	<i>Glyma.18G293200</i>	Drug transmembrane transport; multidrug resistance protein
	<i>Glyma.18G293300</i>	Drug transmembrane transport; multidrug resistance protein

^a model gene retrieved on reference genome V2 from SoyBase (<https://www.soybase.org/> accessed on 4 April 2024). ^b Gene ontology of the retrieved genes from SoyBase (<https://www.soybase.org/> accessed on 3 April 2024).

4. Discussion

The objective of this study was to employ a high level of intraspecific mapping of ZM6 and MN populations, analyzed for two different periods, to find stable QTLs (QTL Hotspots) and to determine probable candidate genes associated with soybean's resistance to *C. ilicicola*. We found six QTL hotspots and predicted 23 potential candidate genes (PCGs).

4.1. Examining the Resistance to RCR in Two RIL Populations

Due to its fast spreading and severity in major production regions, soybean pathologists and breeders have aimed to identify and incorporate RCR resistance into high-quality soybean varieties. Earlier works have developed screening protocols for evaluating soybean germplasm for resistant accession identification [25,58–60]. There are a few sources of incomplete resistance, primarily found in wild relatives or non-elite germplasm. This requires a process of crossing and careful selection, which is made more difficult by environmental factors that affect the pathogen's lifestyle. The ANOVA analysis showed a substantial variation across all the RILs in the ZM6 and MN populations for all three traits, with the environment playing a substantial role (Table S2). The research found that the paternal accessions Zhengyang and NN1138-2 showed a significant resistance level, while Meng8206 and M8180 revealed some level of susceptibility. The disease severity

index analysis revealed that the resistant accessions Zhengyang and NN1138-2 potentially harbor distinct resistance genes. The high correlation coefficients among the traits used for screening RCR resistance imply an independent and interdependent relationship among the traits, suggesting some common loci may be responsible for at least two of the traits studied (Figure 2).

4.2. QTL Detected for Red Crown Rot Resistance in ZM6 and MN Population

The availability of genetic markers is crucial to assist QTL identification. Furthermore, combining QTLs that resist *C. ilicicola* infection within a cultivar is an additional advantage of identifying QTLs connected with soybean RCR resistance. Although some progress has been made in establishing resistance to RCR, data regarding DNA markers associated with resistance to RCR are scarce. Linkage mapping has the potential to facilitate mutual verification and enhance the accuracy of produced results, promoting its application in several studies involving disease resistance gene identification in soybeans using RIL populations [34]. The high-density genetic maps for ZM6 were genotyped by RAD-seq technology [32], and the MN population was also genotyped by SLAF technology [61]. The markers on both linkage maps, ZM6 and MN, were incorporated into all the 20 linkage groups and covered a length of 2630.22 cM and 2054.50 cM, respectively. In ZM6, the average distance among adjacent markers was 1.01 cM, while in MN, it was 1.00 cM. By combining these high-density genetic maps, there is potential to accurately locate QTL that has significant and subtle effects on resistance to RCR in the mapping populations.

The capacity to detect QTL determines the extent of the genomic region, which is decided by the gene variation between parental individuals and is influenced by population structure and size [62,63]. The two RIL populations identified 15 and 14 QTLs associated with RCR resistance in ZM6 and MN populations, respectively, totaling 29 QTLs. For the ZM6, the most substantial effect was on chromosome 7, *qSR-7-2zm6*, with LOD 5.19, while for MN, the highest was on chromosome 1, *qER-1-1mn* with a LOD of 7.61 (Tables 3 and 4). This confirms that the phenotypic data gathered in different settings exhibit statistical differences [64]. The identification of QTL for resistance to RCR provides evidence that quantitative traits are impacted by multiple QTLs, each with relatively small individual effects. Hence, we hypothesize that resistance to RCR is a complex genetic characteristic regulated by several resistance gene loci. For instance, in soybeans, several QTLs are identified for *S. sclerotiorum* and *P. sojae* resistance, confirming that their genetic architecture is influenced by several genes [7,65,66].

The QTLs *qER-7-1zm6*, *qER-10-2zm6*, *qER-11-1zm6*, *qSR-7-1zm6*, *qSR-7-2zm6*, and *qSR-11-1zm6* in the ZM6 accounted for phenotypic variation above 10% and were on Chr07, Chr10, or Chr11. Therefore, the desired alleles of the genetic markers can significantly decrease the disease traits of RIL progenies and alter the response characteristics of *C. ilicicola*. Also, the MN population had eight QTLs with a PVE of more than 10%, of which *qER-1-1mn* had its PVE of 25.24% on Chr01. Thus, our findings demonstrated the dependability of QTL mapping. Furthermore, QTLs could be utilized as main targets for discovering candidate genes and implementing marker-assisted selection in future studies. The results prove that RIL has segregated homozygous alternative alleles and can detect QTL due to the presence of half alleles in the RIL [67].

4.3. Identification of QTL Hotspot for Resistance to RCR in Soybean

The durability of the QTL is essential for its application in a breeding strategy. This study additionally showed the localization of QTLs on chromosomes for many variables (ER, SR, and DS) associated with RCR resistance. For instance, in the two environments, we confirmed the presence of six (6) QTL hotspots for resistance to RCR from the ZM6 and MN RIL populations on chromosomes 1, 7, 10, 11, 13, and 18 (Table 5). Hotspot E consisted of *qDS-13-1zm6* and *qDS-13-1mn*, which were obtained from both populations colocalized in the same physical interval. Our investigation found that the *qSR-7-2zm6* and *qDS-18-2zm6* genes in the ZM6 population were localized in the same area on chromosome 7 for two

consecutive years (Table 5). These findings indicate that there may be a shared resistance mechanism to several RCRs in soybeans, and a specific gene on chromosome 7 could be responsible for providing resistance to RCR. We have a firm conviction that these QTLs have the potential to be significant QTLs for either gene cloning or marker-assisted selection.

Determining the precise gene(s) accountable for resistance loci is an essential stage in the ongoing endeavor to examine the molecular processes and biological basis of quantitative resistance to diseases. The gene ontology (GO) study revealed that the majority of genes in the six hotspots were mainly involved in cellular functions such as the cell, cell component, organelle, cellular process, metabolism process, catalytic activity, binding, and transporter activity. These substances are crucial for the growth and development of plants, and multiple studies have verified their role in safeguarding plants from diseases caused by pathogens. To illustrate, the cells, cell parts, and organelles of plants participate in the hypersensitive response in plants due to gene-for-gene resistance. For instance, the cells located around the area where the fungus enters experience a process of programmed cell death (PCD) [68,69], cellular processes, metabolic processes [70] and catalytic activity, binding, and transporter activity [71–73] and are involved in plant immunity in order to impede the progression of the disease.

At the physical position of the six “QTL Hotspots”, 1279 gene models were determined. Among these, 111 genes were identified as PCGs based on the study of GO enrichment, gene function, and existing literature. The actions of these 111 genes engage in activating the plant’s immune response during a pathogen attack. Also, some of the genes activate secondary metabolite processes (lignin biosynthetic, flavonoid biosynthetic, and phenylpropanoid metabolic) as well as signal transduction. Of the 111 predicted PCGs, 23 are noted to provide resistance against *C. ilicicola* invasion in soybeans based on their gene expression data obtained from RNA-seq. Among the 23 PCGs, seven genes, namely, *Glyma.01G112300*, *Glyma.01G126600*, *Glyma.01G127100*, *Glyma.01G127200*, *Glyma.01G127700*, *Glyma.13G076200*, and *Glyma.18G287000*, are reported to offer resistance to crops upon pathogen infection either by activating defense response, signal transduction, or leucine-rich repeat responses/processes. For instance, in soybean, a gene annotated as defense response governs tolerance to *Phytophthora* root rot disease [74], while leucine-rich repeat has the highest number of documented disease resistance genes [75]. Similarly, *Glyma.07G133900* and *Glyma.07G134100* encode multicopper oxidase. In wheat, ascorbate oxidase and skewed5-similar proteins resulting from multicopper oxidase partake in the plant’s immunity, and their silencing promotes the plant’s immunity to *Verticillium wilt* [76]. The soybean *GmHSP40* results in cell death and enhances its immunity to mosaic virus, as its silencing results in making soybean more sensitive to the virus [77]. Similar roles are confirmed in pepper by overexpressing *CaHSP70a* [78]. Hence, *Glyma.18G289100* and *Glyma.18G289600* encoding the Hsp70 protein could possibly function to regulate RCR resistance.

We anticipated that the 23 predicted candidate genes may have a potential role in regulating RCR resistance in soybeans. However, additional testing and verification are necessary to demonstrate their specific involvement in soybean resistance to RCR, as well as their possible application in disease breeding programs. Also, the identified loci and QTL hotspot will aid in validating *C. ilicicola* resistance in soybeans through systematic breeding. Additionally, these genomic areas can serve as targets for enhancing our understanding of the RCR mechanism and improving soybean resistance to *C. ilicicola*. The identified harbor QTLs demonstrating promise will be employed for precise mapping and molecular cloning of crucial loci in the future. These regions can then be utilized to improve soybean plant immunity to *C. ilicicola* invasion.

5. Conclusions

The study examined the genetic architecture of soybean resistance to *C. ilicicola* invasion. A total of 29 QTLs were identified via linkage mapping using two different RIL populations. All the detected QTLs were grouped into six significant “QTL hotspots” and

represent the major and consistent genomic areas that control the inheritance of soybean resistance to RCR. Among them, QTLs *qER-1-1mn* and *qSR-7-2zm6* accounted for 25.24% and 14.87% of the overall phenotypic variation, respectively. A common QTL *qDS-13-1zm6* and *qDS-13-1mn* were obtained from both populations colocalized in the same physical interval. We spotted 23 genes likely to regulate RCR resistance based on the six genomic areas known as “QTL hotspots”. The identified genes serve as a ground for future research in developing genetic resources for enhancing soybean resistance. Similarly, additional functional studies are required for the validation and cloning of functional genes of the proposed candidate genes to determine their exact functions in regulating RCR resistance. These findings establish a crucial basis for generating fungal-resistant QTL for RCR in soybeans through cloning.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy14081693/s1>, Table S1: Distribution of bin and SLAF markers mapped on soybean chromosomes/linkage groups in ZM6 and MN Populations. Table S2: Combined analysis of variance (ANOVA) for RCR evaluation trait (ER, SR, and DS) in ZM6 & MN RIL Population across two time screening (1st and 2nd time screening). Table S3: Model genes within hotspot A, hotspot B, hotspot C, hotspot D and hotspot E regions in both RIL Populations (ZM6 & MN). Table S4: Predicted candidate genes within hotspot A, hotspot B, hotspot C, hotspot D and hotspot E regions in both RIL Populations (ZM6 & MN) based on known functional annotation. Table S5: DEGs following infection by *C. ilicicola* Kobayashi et al., 2022 [54]. Figure S1: AgriGO annotation Palatino Linotype for the QTL Hotspot A and B. (A) QTL Hotspot A, (B) QTL Hotspot B. Figure S2: AgriGO annotation information for the QTL Hotspot C and D. (C) QTL Hotspot C, (D) QTL Hotspot D. Figure S3: AgriGO annotation information for the QTL Hotspot E and F. (E) QTL Hotspot E, and (F) QTL Hotspot F. Figure S4: Heat map displaying the expression patterns of 23 selected genes in various soybean tissues from six QTL hotspots.

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