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Generation of a High-Density Genetic Map of Pepper (*Capsicum annuum* L.) by SLAF-seq and QTL Analysis of *Phytophthora capsici* Resistance

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Abstract: Pepper (*Capsicum annuum* L.) is an economically significant global crop and condiment. Its yield can be severely reduced by the oomycete plant pathogen, *Phytophthora capsici* (*P. capsici*). Here, a high-density genetic map was created with a mapping panel of F_2 populations obtained from 150 individuals of parental lines PI201234 and 1287 and specific-locus amplified fragment sequencing (SLAF) that was then utilized to identify loci that are related to resistance to *P. capsici*. The sequencing depth of the genetic map was 108.74-fold for the male parent, 126.25-fold for the female parent, and 22.73-fold for the offspring. A high-resolution genetic map consisting of 5565 markers and 12 linkage groups was generated for pepper, covering 1535.69 cM and an average marker distance of 0.28 cM. One major quantitative trait locus (QTL) for the *P. capsici* resistance (*CQPc5.1*) was identified on Chr05 that explained the observed 11.758% phenotypic variance. A total of 23 candidate genes located within the QTL *CQPc5.1* interval were identified, which included the candidate gene *Capana05g000595* that encodes the RPP8-like protein as well as two candidate genes *Capana05g000596* and *Capana05g000597* that encodes a RPP13-like protein. Quantitative reverse-transcription PCR (qRT-PCR) revealed higher expression levels of *Capana05g000595*, *Capana05g000596*, and *Capana05g000597* in *P. capsici* resistance accessions, suggesting their association with *P. capsici* resistance in pepper.

Keywords: pepper; *Capsicum annuum*; *Phytophthora capsici*; high-density genetic map; QTL

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1. Introduction

Pepper (*Capsicum annuum* L.) is a common condiment and an economically significant vegetable crop. It is not only used in many cuisines but also found to have many medicinal properties. In 2019, approximately 212.04 million tons of chilies and peppers were grown on about 49.31 Mha around the world (http://www.fao.org/faostat/zh/#data/QC). However, pepper is susceptible to a variety of pathogens such as CMV, TMV, *Colletotrichum* spp., and *Phytophthora capsici* (*P. capsici*) [1–4]. *Phytophthora* blight can significantly decrease pepper yield and quality [5]. The disease is caused by the oomycete plant pathogen *P. capsici* that initially infects the roots and crown roots, then subsequently spread to every plant part, including the roots, stems, fruits, and leaves [6]. *Phytophthora* blight is a severe disease that commonly occurs under warm (25–28 °C) and highly humid conditions [7–9]. No effective and safe measures to control *Phytophthora* blight have been established to date, except for chemical control [10–13]. Therefore, the utilization of resistant varieties has become a simple, effective, and safe way of resolving *Phytophthora* blight occurrence in pepper. Plant breeders have also focused on selecting varieties with high levels of resistance.

The three physiological races of *P. capsici*, named "races 1–3," have been determined by their virulence on four pepper varieties: early calwonder (sensitive), PI201234 (resistant),

PBC137 (partially resistant), and PBC602 (partial resistance) [14]. Previous studies have reported several pepper accessions that are resistant to *P. capsici*, including PI123469, PI201232, PI201234, AC2258, and CM334 (Criollo de Morelos 334) [4,14–18]. Resistance to *P. capsici* is mainly regulated by a single dominant gene in PI201234 or by one dominant gene in the presence of modifiers [9,19–21], and AC2258, which has been derived from PI201234, is resistant to *P. capsici* [17,18]. Studies have shown that resistance to *P. capsici* in CM334 is controlled by a minimum of two genes [22,23]. In addition, these reports revealed that the regulatory mechanism underlying *P. capsici* resistance in pepper is highly complex. Numerous reports have investigated the effect of a pepper QTLs on chromosomes that are associated with resistance against *P. capsici* [18,23–31]. *Pc5.1* is a homologous QTL on chromosome 5 of CM334, PI201234, and Perennial that has been associated with resistance to *P. capsici* [23,29,31]. Mallard et al. (2013) have identified resistance QTLs among three meta-QTLs (*MetaPc5.1*, *MetaPc5.2*, and *MetaPc5.3*) by meta-analysis [31]. Siddique et al. (2019) identified three QTLs on chromosome P5, including *QTL5.1*, *QTL5.2,* and *QTL5.3*, which were associated with resistance to three *P. capsici* isolates (race 1, race 2, and race 3) by traditional QTL mapping combined with GWAS strategy [30]. In addition, a few minor-effect QTLs has been identified on different chromosomes [23,27,28,32].

Large-scale SNP markers have recently been discovered by next-generation sequencing (NGS) that have expedited the construction of the pepper genetic map. SLAF-seq is a novel high-throughput sequencing technique that is less expensive and complex than high-quality reference genome libraries [33]. In addition, the SLAF-seq strategy has been generally utilized in constructing high-density genetic maps of different species and in QTL mapping [34–42]. This strategy had also been successfully used in the creating high-density pepper genetic maps [40,43,44]. For instance, Guo et al. (2017) determined two candidate CMV resistance genes on pepper chromosomes 2 and 11 using SLAF-seq along with BSA technologies [43]. In addition, Zhang et al. (2019) utilized SLAF-seq in detecting two major QTLs that were strongly associated with FFN [40].

In this work, we developed a high-density pepper linkage map with SLAF-seq as well as identified QTLs that are related to *P. capsici* resistance using F_2 populations that were obtained from a cross between parental lines 1287 (*P. capsici* susceptible, female) and PI201234 (*P. capsici* resistant, male). Finally, we investigated the main effect of QTLs as well as select candidate genes. Our results could potentially facilitate the elucidation of the genetic mechanism underlying *P. capsici* resistance in pepper and lay the foundation for breeding highly resistance pepper cultivars.

2. Materials and Methods

2.1. Mapping Population

The *P. capsici*-susceptible sweet pepper line "1287" was obtained from Zhongjiao808, whereas the *P. capsici*-resistant "PI201234" was collected from Central America. The present study used an F_2 mapping population, comprising 150 individuals that were obtained by crossing female parent 1287 and male parent PI201234, which was then used as mapping population. The parental lines and the F_2 population were grown at the Chongqing experimental station of the Chongqing Academy of Agricultural Sciences (Chongqing, China). Sowing of pepper seeds was performed using 50-cell trays containing a mixture of peat and vermiculite that was autoclave sterilized for 30 min in 2018.

2.2. Pathogen Preparation and Plant Inoculation with P. capsici

P. capsici isolate HT1 was used for *P. capsici* resistance identification in pepper. HT1 has been identified as physiological race 3 and was isolated from infected pepper fruit at the experimental station in Jiulongpo District, Chongqing, China. The isolate was cultured on V8 juice-agar medium at 28 °C in an incubator. To prepare the inoculums for disease screening, the cultures were soaked in 5 mL ddH_2O and cultivated at 4 °C for 1 h and then set at room temperature for 1 h to promote sporulation. Spore density was determined using a hemocytometer and adjusted to 1×10^5 spores/mL in distilled water. Before

inoculation, pepper plants were soaked in water. Then, 5 mL of suspension was injected into the root of each six- to seven-true-leaf stage pepper plant. The inoculated plants were then grown at 28 °C for 16 h/day and at 80% relative humidity.

2.3. Disease Evaluation

Seven days post inoculation, the plants were assessed for disease symptoms using the 0–5 scale of the Chinese standard NY/T 2060.1-2011 (Ministry of Agriculture of the People's Republic of China 2011), which consisted of the following: 0 = no disease symptoms; 1 = emergence of brown lesions in the roots and stems with no to slight wilting of leaves; 2 = extension of root and stem lesions by 1–2 cm, the leaves wilted and had fallen off; 3 = root and stem lesions exceed 2 cm and leaves clearly show wilting or defoliation; 4 = large brown lesions on stems are extended and dehydrated, with the exception of the uppermost leaves which have been lost; and 5 = plant death. According to the disease grade of each plant, the disease index (DI) of each identification material was calculated. The DI was calculated using the equation below:

$$DI = \frac{\Sigma(s \times n)}{N \times S} \times 100,$$

where s is the disease level ranging between 0 and 5; n is the number of plants with corresponding disease level; N is the number of plants investigated in each F_2; and S is the representative value of the highest grade.

2.4. Statistical Analysis of Phenotypic Data

The laboratory study was conducted at the experimental station of the Chongqing Academy of Agricultural Sciences. The phenotypic data collected for the disease parameters were considered and analyzed as individual traits. The resistance traits were recorded for the F_2 population and parents. The traits means were calculated using DPS 18.10 (DPS, China).

2.5. DNA Extraction, SLAF Library Construction, and High-Throughput Sequencing

An improved CTAB method was utilized to extract genomic DNA from the young leaves of two parental lines and 150 F_2 individuals that were at the five- to six-leaf stage [45]. We employed an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA) and performed 1.0% agarose gel electrophoresis to respectively measure DNA concentration and quality. The SLAF-seq library was constructed as detailed previously by Sun et al. [33], with only a few small changes. The restriction enzyme *Hae*III (New England Biolabs, NEB, USA) was utilized for digestion of the genomic DNA of the parental lines and individuals of the F_2 population. We added polyA tails to the 3′ ends of the digested fragments, which were then connected to duplex-labelled sequencing adapters and PCR amplified. PCR was performed with the diluted restriction-ligation DNA sample, Q5® High-Fidelity DNA polymerase (NEB), dNTPs, and PCR primers (forward, 5′-AATGATACGGCGACCACCGA-3′ and reverse, 5′-CAAGCAGAAGACGGCATACG-3′). The PCR products were purified with Agencourt AMPure XP beads (Beckman Coulter, High Wycombe, UK) and then resolved on a 2% agarose gel. Fragments that were 314 to 364 bp in size were separated and purified with a QIAquick gel extraction kit (Qiagen, Hilden, Germany). SLAF-seq was then conducted on an Illumina High-Seq 2500 sequencing platform (Illumina, San Diego, CA, USA) at Beijing Biomarker Technologies Corp. (Beijing, China, http://www.biomarker.com.cn, accessed on: 8 January 2019). We employed the *Oryza sativa* L. genome as reference for quality control and conducted library construction and sequencing using similar settings as that for the pepper mapping population.

2.6. SLAF-seq Data Grouping and Genotyping

In this study, reads with a quality score below Q30 (quality score < 30e) were filtered out. After that, high-quality reads were mapped to the pepper reference genome utilizing

BWA software, with the paired-end mapped reads at the identical position and >95% identity divided into a single SLAF locus. In each SLAF, a polymorphism locus was observed between the parents, of which most were SNPs. All of the polymorphism SLAF loci were then genotyped with consistency at SNP loci of the offspring and parents. SLAFs that consisted of more than eight SNPs were screened out, and then the parental SLAFs with a sequencing depth of <10-fold were discarded. A high-density linkage map was then created using polymorphic SLAFs showing parental homozygosis (aa × bb).

2.7. High-Density Linkage Map Construction

We quantified the modified logarithm of odds (MLOD) value between two adjacent markers and markers with MLOD values < 5 were filtered out. Then, the SLAF markers were assigned to chromosomes (Chr), and 12 Chr were obtained. Simultaneously, we analyzed the linear array of markers in every Chr using HighMap software [46] and then estimated the genetic distances between a pair of adjacent markers.

2.8. QTL Mapping of P. capsici Resistance and Candidate Gene Prediction

QTL analysis was identified by r/QTL software using CIM methods [47,48]. The LOD score thresholds for evaluating the statistical significance of the QTL effects were established using 1000 permutations ($p < 0.05$). The predicted genes within the target QTL interval were determined by comparison with the annotated Zunla-1 and CM334 reference genomes (http://peppersequence.genomics.cn, accessed on: 20 January 2019). The function of genes identified in the candidate regions was manually determined by BLASTX (https://blast.ncbi.nlm.nih.gov/, accessed on: 20 January 2019). In addition, the predicted genes were further annotated based on KEGG (https://www.kegg.jp/kegg/, accessed on: 20 January 2019), COG (http://www.ncbi.nlm.nih.gov/COG/, accessed on: 20 January 2019), Swiss-Prot (http://www.ebi.ac.uk/uniprot/ accessed on: 20 January 2019), and NR (https://blast.ncbi.nlm.nih.gov/ accessed on: 20 January 2019) databases.

2.9. qRT-PCR Analysis

For expression analysis, we conducted qRT-PCR to investigate the expression pattern of five disease-resistant or defense-related genes for *P. capsici* resistance in pepper. Leaf samples were gathered from days 0, 1, 2, 3, 4, 5, 6, and 7 post inoculation with *P. capsici* in the resistant line "PI201234" and the susceptible line "Early calwonder." "Early calwonder" was defined as susceptible to three physiological races of *P. capsici*. Total RNAs were extracted utilizing the Plant RNA Kit (Tiangen DP441, China) as per the company's instructions. Subsequently, cDNAs were reverse-transcribed using TaKaRa Reverse Transcription Kit (Takara Biomedical Technology (Beijing) Co., Ltd., Beijing, China). Quantitative PCR was conducted on a 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) using TB Green® Premix Ex Taq™ Kit (TaKaRa). The PCR program was as follows: Holding Stage Step 1: 95 °C 30 s, followed by 40 cycles of Step 1: 95 °C for 5 s, Step 2: 60 °C for 30 s, and 72 °C for 2 min. After the last cycle, the amplification was extended for 7 min at 72 °C. *AY572427* was used as internal control for qRT-PCR analysis. We employed the $2^{-\Delta\Delta T}$ method to determine relative expression levels of candidate genes, which were normalized to that of actin gene *(AY572427).* Each target sample was analyzed using three biological replicates. All values were reported as the mean ± standard deviation (n = 3), and the statistical significance of any differences was analyzed using a Student's *t*-test.

3. Results

3.1. Sequencing and Genotyping Based on SLAF-seq

In this study, genotyping of 150 F_2 individuals and their parents was performed using the SLAF-seq technology. The sequencing data generated in this work were sent to the NCBI SRA database (http://www.ncbi.nlm.nih.gov/sra/ accessed on: 20 October 2020) as accession no. PRJNA669602. Approximately 76.22 GB of raw bases and 381.15 Mb of paired-end reads were generated, of which 94.37% achieved or exceeded quality score of 30

(Q30), and GC (guanine-cytosine) content was 38.86% (Table 1). *Oryza sativa* L. was used as control for evaluating the effectiveness of library construction. In addition, 12,250,440 reads representing 139,046 SLAFs with average depths of 63.83 were obtained from the male parent (PI201234), and 13,232,257 reads representing 141,584 SLAFs with average depths of 72.32 were obtained from the female parent (1287) (Table 1). In the offspring (F_2 population), 2,371,153 reads that were representing 124,582 SLAFs with average depths of 14.66 were generated (Table 1).

Table 1. Specific-locus amplified fragment sequencing (SLAF)-seq data statistics of the *Capsicum* F_2 population.

Samples	Total Read	Total Bases	Q30 Percentage (%)	GC Content (%)	SLAF Number	Total Depth	Average Depth(X)
PI201234	12,250,440	2,449,757,552	93.98	38.42	139,046	8,875,578	63.83
1287	13,232,257	2,646,335,364	94.71	38.15	141,584	10,239,208	72.32
Offspring	2,371,153	474,202,396	94.37	38.36	124,582	1,825,928	14.66
Total	381,155,587	76,226,452,308	94.37	38.86	405,212	/	/

After filtration of low-depth SLAF tags, approximately 174,193 high-quality SLAF markers were obtained, of which 19.77% (34,432) were polymorphic SLAFs (Table 2). In addition, 25,839 of the 34,432 polymorphic SLAFs were cultured into eight segregation patterns (aa×bb, ab×cc, ab×cd, cc×ab, ef×eg, hk×hk, lm×ll, and nn×np) (Figure 1). As the parents were homozygous (i.e., with genotype aa or bb), 21,069 SLAFs exhibited the aa×bb segregation pattern and were successfully selected for map construction.

Table 2. Description on basic characteristics of the 12 linkage groups.

Linkage Group	SLAF Number	Polymorphic
Chr01	16,109	3221
Chr02	9259	1626
Chr03	15,231	3159
Chr04	12,696	1569
Chr05	13,024	2986
Chr06	12,887	2640
Chr07	11,667	1907
Chr08	9426	1263
Chr09	14,507	3250
Chr10	11,356	1687
Chr11	11,890	3937
Chr12	13,089	2343
Other	23,052	4844
Total	174,193	34,432

3.2. Genetic Map Construction

After four-step filtering, our final map contained 5565 markers on 12 Chrs, which were designated Chr01-Chr12 using HighMap software and presented in Table 2 and Figure 2. The linkage map encompassed a total of 1535.69 cM and exhibited an average marker distance of 0.28 cM (Figure 2). The largest Chr was Chr03, which consisted of 444 markers, showed a length of 169.18 cM, and an average marker-to-marker distance of 0.38 cM, while the smallest Chr was Chr05 that consisted of 460 markers, showed a length of 99.98 cM, and an average marker-to-marker distance of 0.22 cM (Table 3). The extent of linkage between markers was represented by the percentage of "Gaps $\leq$ 5 cM," which ranged from 99.08% to 100%, and an average of 99.70% (Table 3). The largest gap on this linkage map was situated on Chr10 at 9.99 cM, whereas the smallest gap was 3.94 cM at Chr08 (Table 3).

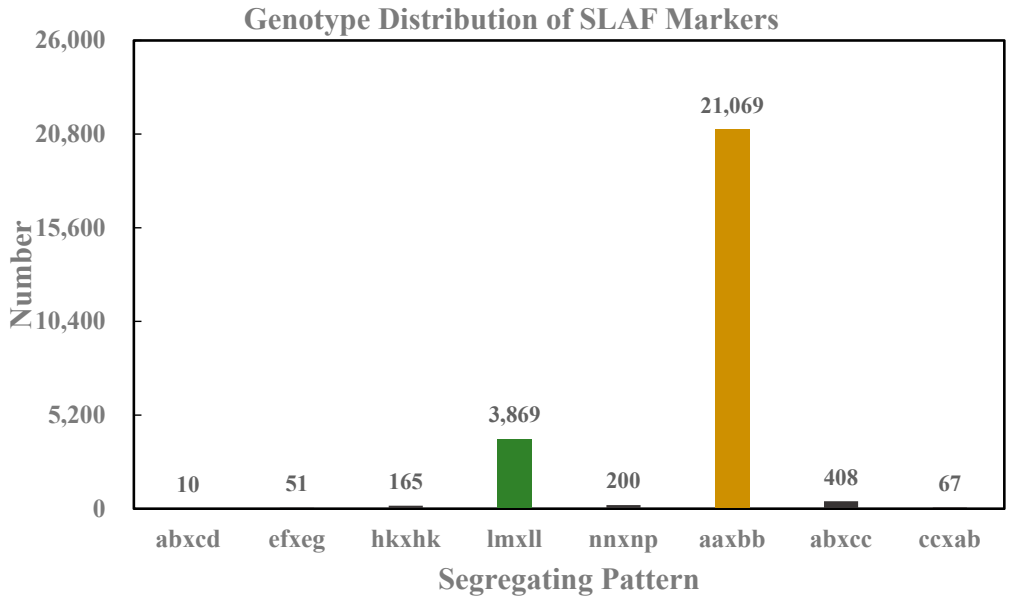

Figure 1. Specific-locus amplified fragment sequencing (SLAF) polymorphism analysis. Marker count in eight segregation patterns. The *x*-axis represents eight segregation patterns for the polymorphic SLAF markers, and the *y*-axis shows the number of markers.

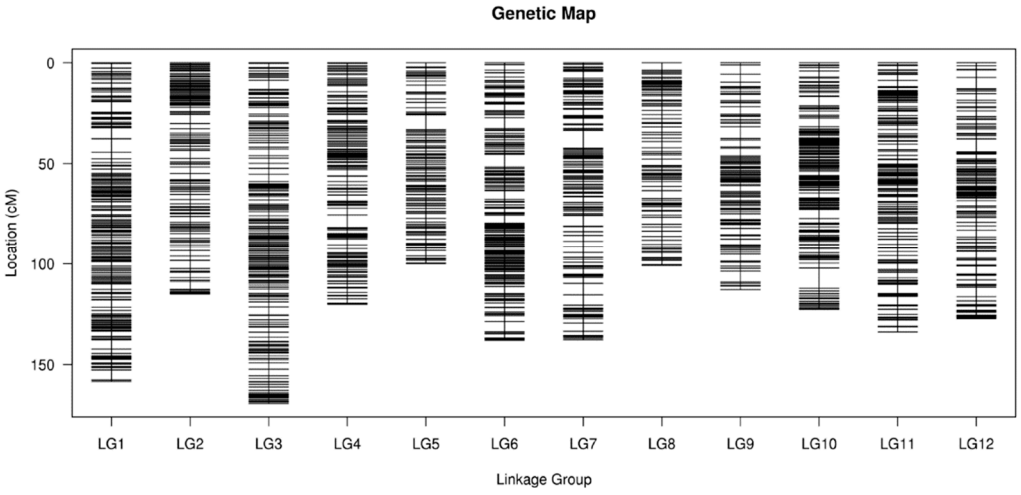

Figure 2. Distribution of SLAF markers across 12 pepper linkage groups. The black bar indicates a SLAF marker. The *x*-axis shows the linkage group number, whereas the *y*-axis represents genetic distance.

3.3. Quality and Accuracy of the Genetic Map

The quality and accuracy of the genetic map were assessed based on collinearity between the genetic and physical maps. The average integrity of each marker was 99.91% (Figure 3). Furthermore, among the 12 linkage groups, Chr03 showed the highest collinearity, with a correlation coefficient of 0.9979, and the average Spearman's rank correlation coefficient was 0.9758 (Table 3). On average, the coverage of these markers was 108.74-fold in PI201234 (male parent), 126.25-fold in 1287 (female parent), and 22.73-fold in every F_2 individual (Table 4), thereby indicating genotyping accuracy. Furthermore, collinearity with the physical map was utilized to examine the quality of the genetic map. Figure 4 shows that most of the genetically mapped loci were collinear with their physical positions on the reference genome sequence of *C. annuum* cv. Zunla-1 v2.0 [45]. Every correlation coefficient of 12 linkage groups was also assessed. The correlation coefficients of the 12 linkage groups

were all close to 1, which indicated relatively high collinearity between linkage groups and the pepper reference genome (Figure 3).

Table 3. Basic information of the 12 linkage groups. The closer the Spearman's rank correlation coefficient is to 1, the better the collinearity.

Linkage Group	Marker Number	Average Distance between Markers (cM)	Size (cM)	Gaps ≤ 5	Max Gap (cM)	Correlation Coefficient
Chr01	437	0.36	158.32	99.08%	6.77	0.9968
Chr02	415	0.28	114.98	100.00%	4.57	0.9273
Chr03	444	0.38	169.18	100.00%	4.63	0.9979
Chr04	336	0.36	120.20	99.70%	6.38	0.9948
Chr05	460	0.22	99.98	99.78%	7.29	0.9054
Chr06	722	0.19	137.92	99.58%	6.31	0.9977
Chr07	517	0.27	137.66	99.22%	8.71	0.9712
Chr08	373	0.27	100.81	100.00%	3.94	0.9568
Chr09	414	0.27	112.88	99.52%	5.82	0.9875
Chr10	532	0.23	122.63	99.81%	9.99	0.9963
Chr11	458	0.29	133.80	100.00%	4.70	0.9974
Chr12	457	0.28	127.33	99.56%	6.38	0.9803
Maximum	722	0.38	169.18	100.00%	9.99	0.9979
Minimum	336	0.19	99.98	99.08%	3.94	0.9054
Total	5565	0.28	1535.69	/	/	/
Average	463.75	/	127.97	99.70%	/	0.9758

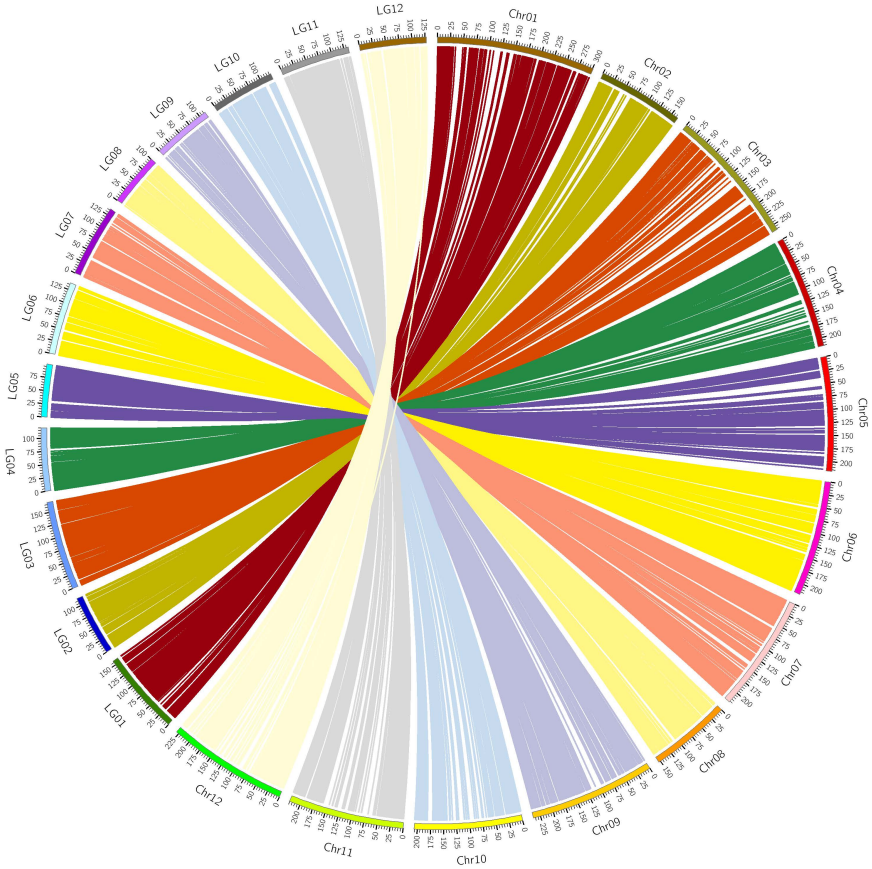

Figure 3. Collinearity between genetic and physical maps. The correlation between the pepper chromosomes (Chr) and the linkage group (LG) of the genetic map is illustrated.

Table 4. Details on the depth of mapped markers.

Samples	Marker Numbers	Total Depth(X)	Average Depth(X)
PI201234	5565	605,153	108.74
1287	5565	702,568	126.25
Offspring	5513	125,293	22.73

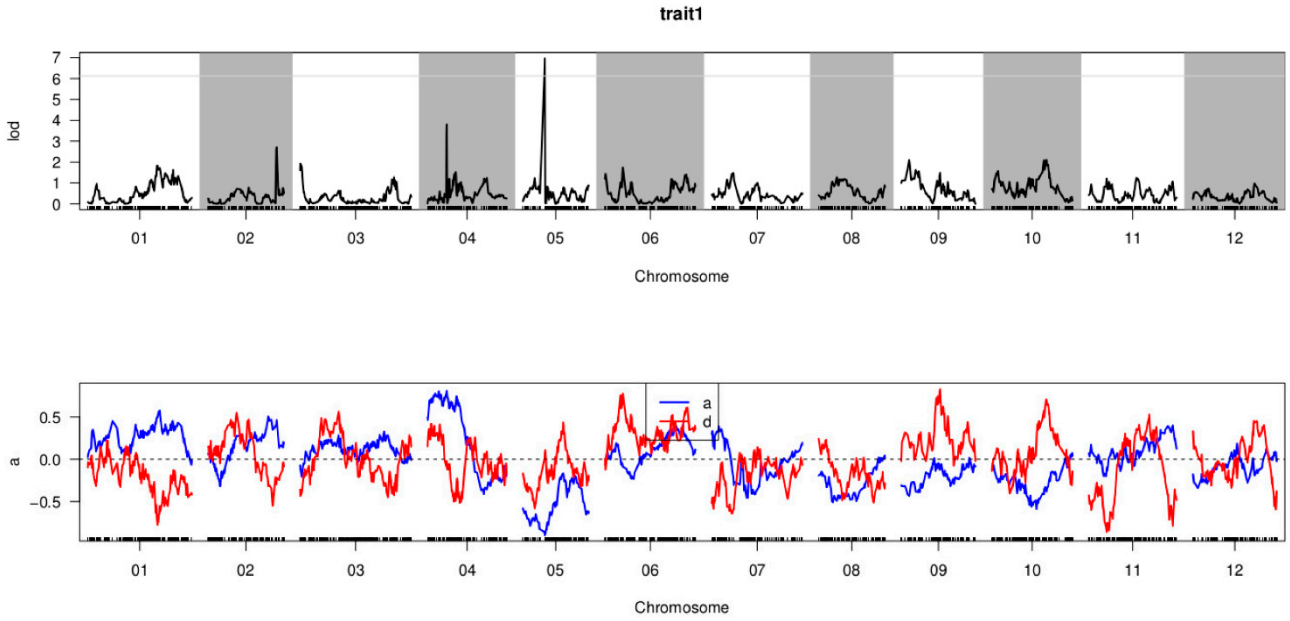

Figure 4. Quantitative trait locus (QTL) analysis of the *P. capsici* resistance trait of pepper. The *x*-axis indicates linkage group in pepper, and the *y*-axis presents LOD values. The blue line indicates the additive effect, and the red line represents the dominant effect.

3.4. Phenotypic Analysis of P. capsici Resistance

In 2008, the disease indices (DIs) of 150 F_2 populations were determined (Table 5). The highest DI value was recorded in the susceptible 1287 (84.3), while the lowest ID value was recorded in the resistance parent PI201234 (7.9). The DI values of the F_2 population varied between 0.00 and 100. The average DI value of the F_2 population was 47.2. The variation was 0.75, and skewness and kurtosis value of the DI in the F_2 population was small, indicating that the population was suitable for QTL identification.

Table 5. Descriptive statistics of disease index and the whole population of the parents.

Traits	F_2 Population								Parent		
	Min	Max	Range	Average	Standard Error	Var	Skew	Kurt	PI201234	1287	Midparent
Disease index (100%)	0	1	0–1	0.4772	0.029	0.75	−0.17	−1.43	7.9	84.3	46.1

3.5. QTL Mapping of P. capsici Resistance

In present study, the maximum LOD value of 6.972 was used as the threshold to determine the existence of QTL. Based on the high-density genetic map, a single major QTL for the *P. capsici* resistance trait was identified in the F_2 population and designated as *CQPc5.1* (Table 6, Figure 4), which explained 11.76% of the observed phenotypic variance. *CQPc5.1* was localized in 17.9–19.4 Mb on Chr05, which encompassed a genetic distance of about 0.35 cM, as well as a physical distance of about 1.47 Mb on Chr05 (Table 6).

Table 6. Quantitative trait locus (QTL) analysis of the *P. capsici* resistance trait in F_2 populations.

QTL	LOD Threshold	Chr ID	Physical Distance Interval (bp)	Genetic Distance Interval (cM)	Max LOD	ADD	DOM	PVE (%)
CQPc5.1	6.125	05	17,967,630–19,446,349	33.103–33.448	6.972	−0.897	0.079	11.758

Note: LOD, logarithm of odds. Maximum LOD score (QTL peak). ADD, additive effects. DOM, dominance effects. PVE, phenotypic variance explained.

3.6. Candidate Gene Prediction and qRT-PCR Analysis

According to the annotations of the *C. annuum* cv. Zunla-1 v2.0 genome, 23 predicted candidate genes were determined in the physical interval of *CQPc5.1* on Chr05 (Table 7). Among these, nine candidate genes were identified in the COG database, including 11 genes with KEGG annotations and 12 genes with Swiss-Prot annotations. Furthermore, 5 of the 23 genes were related to disease resistance or defense, and thus might be involved in *P. capsici* resistance in pepper; the *Capana05g000595* gene was annotated as disease resistance protein, RPP8-like; two genes (*Capana05g000596* and *Capana05g000597*) were annotated as disease resistance protein, RPP13-like; *Capana05g000598* was annotated as likely LRR receptor-like serine/threonine-protein kinase; and *Capana05g000604* was annotated as an F-box/LRR-repeat protein. These five genes were then analyzed by qRT-PCR. The primer sequences are listed in Table 8. The results showed that three genes (i.e., *Capana05g000595*, *Capana05g000596*, and *Capana05g000597*) were up-regulated in "PI201234," and expression levels peaked 2–3 days after pathogen inoculation (Figure 5). Five genes were up-regulated in "Early calwonder" after pathogen inoculation, and expression levels peaked at 5 days. In "Early calwonder," the expression of *Capana05g000604* gradually increased over time; however, it was expressed at a markedly lower level in "PI201234."

Table 7. Details of the annotated candidate genes.

Gene	Start	Stop	COG	KEGG	Swiss-Prot	Nr
Capana05g000592	18,024,840	18,035,001	–	K17550 (protein phosphatase 1 regulatory subunit 7)	Protein phosphatase 1 regulatory inhibitor subunit PPP1R7 homolog	PREDICTED: protein phosphatase 1 regulatory subunit pprA-like
Capana05g000594	18,317,198	18,327,621	–	K13099 (CD2 antigen cytoplasmic tail-binding protein 2)	–	PREDICTED: CD2 antigen cytoplasmic tail-binding protein 2
Capana05g000595	18,357,315	18,357,857	–	–	Disease resistance RPP8-like protein	Hypothetical protein T459_14155
Capana05g000596	18,358,568	18,359,167	General function prediction only	–	Putative disease resistance RPP13-like protein	Hypothetical protein T459_14156
Capana05g000597	18,359,457	18,359,987	–	–	Disease resistance protein RPP13	Hypothetical protein BC332_12877
Capana05g000598	18,387,075	18,390,244	Transcription	–	Probable LRR receptor-like serine/threonine-protein kinase At3g47570	PREDICTED: probable LRR receptor-like serine/threonine-protein kinase At3g47570
Capana05g000599	18,390,817	18,396,058	Carbohydrate transport and metabolism	K05298 (glyceraldehyde-3-phosphate dehydrogenase (NADP+))	Glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic (Fragment)	PREDICTED: glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic
Capana05g000600	18,401,537	18,405,948	General function prediction only	–	Protein high chlorophyll fluorescent 107	PREDICTED: protein high chlorophyll fluorescent 107
Capana05g000601	18,410,078	18,412,165	Post-translational modification, protein turnover, chaperones	K00587 (protein-S-isoprenylcysteine O-methyltransferase)	Protein-S-isoprenylcysteine O-methyltransferase B	PREDICTED: protein-S-isoprenylcysteine O-methyltransferase A-like isoform X1
Capana05g000602	18,413,946	18,415,745	–	–	Pentatricopeptide repeat-containing protein At2g13600 OS = Arabidopsis thaliana	PREDICTED: pentatricopeptide repeat-containing protein At2g13600
Capana05g000603	18,424,439	18,427,748	Coenzyme transport and metabolism	–	FAD synthetase 1, chloroplastic	PREDICTED: FAD synthetase 1, chloroplastic-like
Capana05g000604	18,449,805	18,454,056	Transcription	K10268 (F-box and leucine-rich repeat protein)	F-box/LRR-repeat protein 4	PREDICTED: F-box/LRR-repeat protein 20

Table 7. *Cont.*

Gene	Start	Stop	COG	KEGG	Swiss-Prot	Nr
Capana05g000605	18,709,353	18,710,879	–	–	UPF0481 protein At3g47200	PREDICTED: putative UPF0481 protein At3g02645 isoform X1
Capana05g000607	18,749,830	18,750,435	–	–	–	PREDICTED: uncharacterized protein LOC107870380 isoform X1
Capana05g000608	18,775,198	18,776,266	Cell wall/membrane/envelope biogenesis	K18819 (inositol 3-alpha-galactosyltransferase)	Galactinol synthase 2	Galactinol synthase 2
Capana05g000609	18,823,996	18,827,956	–	–	–	Hypothetical protein CQW23_12126
Capana05g000611	18,835,570	18,835,953	–	K13495 (cis-zeatin O-glucosyltransferase)	Zeatin O-xylosyltransferase	PREDICTED: zeatin O-glucosyltransferase
Capana05g000612	18,851,478	18,852,197	–	K13495 (cis-zeatin O-glucosyltransferase)	Zeatin O-glucosyltransferase	PREDICTED: zeatin O-xylosyltransferase-like
Capana05g000613	18,857,046	18,857,423	–	K13495 (cis-zeatin O-glucosyltransferase)	Zeatin O-glucosyltransferase	Hypothetical protein T459_14175
Capana05g000614	18,879,064	18,879,435	–	–	Putative cis-zeatin O-glucosyltransferase	PREDICTED: zeatin O-xylosyltransferase-like
Capana05g000615	18,879,694	18,880,182	–	K13495 (cis-zeatin O-glucosyltransferase)	Zeatin O-xylosyltransferase	Hypothetical protein T459_14174
Capana05g000617	19,193,439	19,194,854	–	K13495 (cis-zeatin O-glucosyltransferase)	Zeatin O-glucosyltransferase	PREDICTED: zeatin O-glucosyltransferase-like
Capana05g000618	19,354,157	19,355,500	Transcription	–	Receptor-like protein Cf-9	Hypothetical protein T459_14173
Total			9	11	20	23

Note: COG, Clusters of Orthologous Groups. KEGG, Kyoto Encyclopedia of Genes and Genom.

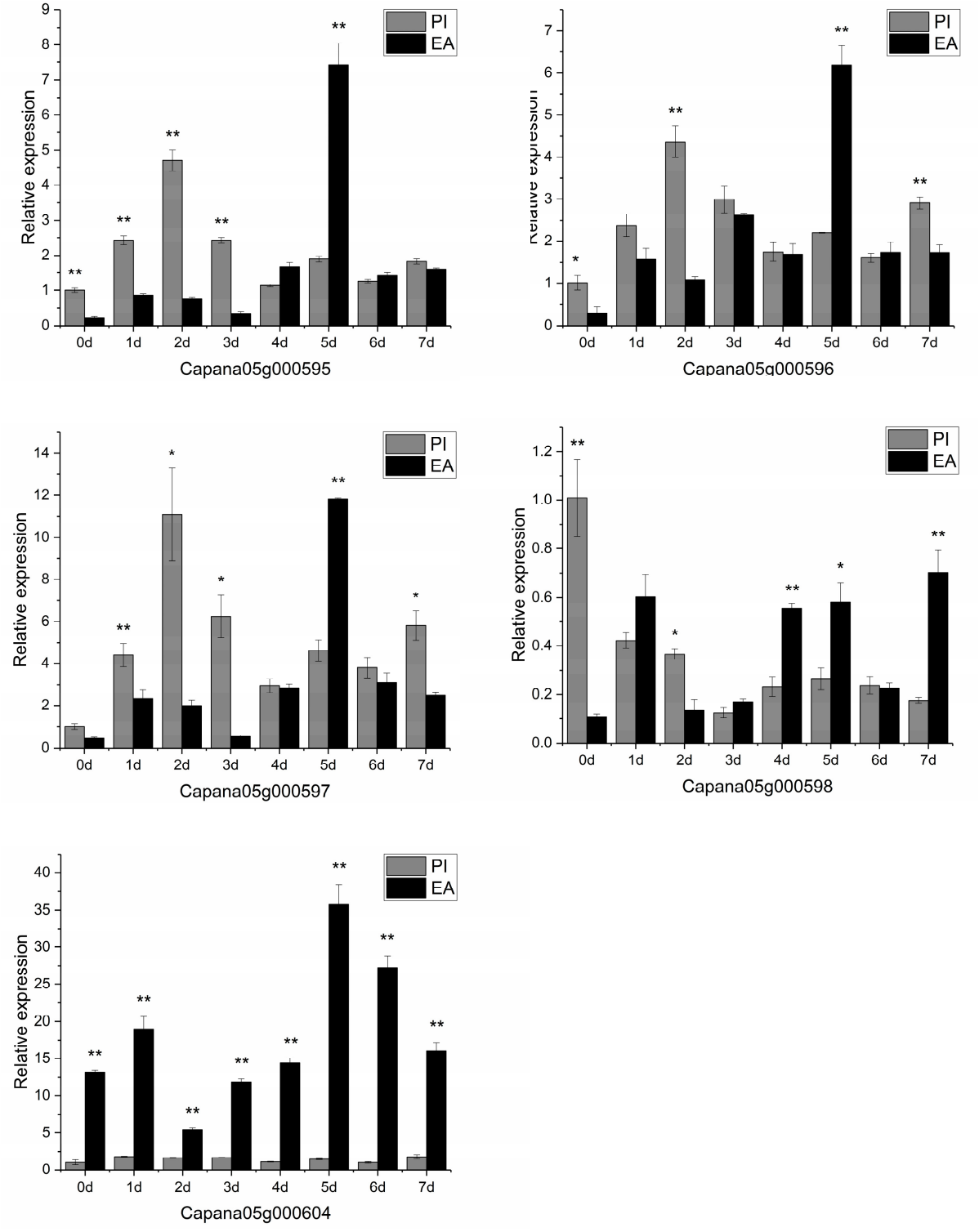

Figure 5. qPCR analysis of five genes in PI201234 and Early calwonder that are related to disease resistance or defense. PI: PI201234 inoculated using *P. capsici* zoospore suspension; EA: Early calwonder inoculated using *P. capsici* zoospore suspension. The *x*-axis shows the time points of sample collection, d: days post-inoculation. The *y*-axis shows the relative expression quantity of genes. Gene expression was normalized to that of actin, and the data were expressed as the mean ± standard deviation of two biological replicates and three technical replicates. A Student's *t*-test was used to analyze statistical significance of differences. * 0.05 level of significance; ** 0.01 level of significance.

Table 8. Information on genes employed in qRT-PCR analysis.

Gene Name	Primer Sequence (5′-3′)	PCR Product Size (bp)	TM (°C)
Capana05g000595	F:AAGGAGGCATTTAGCCGCAA	115	59.0
	R:TGTCTCAAGGCGAGCAACAT		
Capana05g000596	F:CTGCAAGAAAGCGTGTCAGG	98	59.0
	R:AGCCTCCACATCTTTCCACC		
Capana05g000597	F:CAATCCCTCAAGCGACGAGT	121	55.0
	R:CCAGGTCGGACCGATTGTTA		
Capana05g000598	F:ACCTTCCGTGGTGAAATCCC	190	55.0
	R:CGATCCGCGTAACAGGTTTG		
Capana05g000604	F:TTAGCTGTTGCTGAGGGGTG	163	59.0
	R:GCTTGCGTCCAGAGAGACAAA		
Actin (AY572427)	F:AGCAACTGGGACGATATGGAGAAG	198	50.0
	R:AAGAGACAACACCGCCTGAATAGC		

4. Discussion

4.1. Genetic Map Constructed of Pepper

Phytophthora blight caused by *P. capsici* is one of the most serious diseases in pepper, inducing a significant reduction in yield and quality [49]. Despite decades of genetic research on the resistance of pepper to *P. capsici*, no resistant cultivars have been established to date. At present, as a key tool, genetic linkage maps are not only used in plant genetics, but also to identify genomic regions that are related to agronomic and qualitative traits through QTL mapping. Recently, SLAF-seq has been utilized in the creation of genetic linkage maps of pepper, and a number of high-density genetic maps have been successfully created. For instance, Zhu et al. (2019) identified six QTLs using a molecular genetic linkage map via SLAF-seq in relation to flowering time and number of flowers per node in pepper, which consisted of a total of 9038 markers at an average spacing of 0.18 cm that were distributed across 12 linkage groups, and the total distance was 1586.78 cM [44]. In the same year, Zhang et al. (2019) identified two major pepper QTLs (*Ffn2.1* and *Ffn2.2*) that were strongly correlated with FFN using a high-density genetic map, which included 9328 SLAF markers from 12 linkage groups, showing a total genetic distance of 2009.69 cM, as well as an average distance of 0.22 cM [40]. Sun et al. (2020) reported two QTLs that were related to aphid survival (*Rmpas-1*) and reproduction (*Rmprp-1*) using a genetic linkage map that included 167 SNP markers [50]. In this work, we constructed a genetic map using the SLAF-seq technology and according to a F_2 population. The map consisted of 5565 markers that assigned 12 linkage groups, spanning a total length of 1535.69 cM, and showed a mean genetic distance of 0.28 cM. This genetic map exhibited adequate coverage of the polymorphic markers in regions of interest, and the mapped QTLs showed positional accuracy.

4.2. Identification QTL with the Resistance to P. capsici Traits

Previous studies have showed that the major QTLs related to resistance to *P. capsici* are situated on Chr05, despite the use of various resistant lines, pepper populations, or *P. capsici* isolates [23,26,27,32,51]. Mallard et al. (2013) utilized published pepper genome information and identified three major QTLs, namely, *Pc5.1*, *Pc5.2*, and *Pc5.3*, which were localized to the 22.4–24.6, 53.0–162.6, and 9.7–13.3 Mb regions on Chr05, respectively [31]. Siddique et al. (2019) reported three major QTLs on Chr05, namely, *QTL5.1* (18.7–19.5 Mb), *QTL5.2* (27.3–29.2 Mb), and *QTL5.3* (34.6–37 Mb) that were related to resistance to three *P. capsici* isolates on using combined traditional QTL mapping with GWAS [30]. Here, we performed *P. capsici* resistance QTL analysis of pepper. We detected a major QTL *CQPc5.1* based on a high-density linkage map of F_2 plants. *CQPc5.1* was localized to the

17.9–19.4 Mb region on Chr05, with a genetic distance of 33.103–33.448 cM. In an earlier study, Collard et al. (2005) documented that a QTL is only described as "major" when it accounts for >10% of the PVE [52]. In the present study, the phenotypic variance of *CQPc5.1* was 11.58%. In addition, the position of *CQPc5.1* on Chr05 differs from *Pc5.1*, *Pc5.2*, and *Pc5.3*, yet the location of *CQPc5.1* that was identified in this work coincides with that of the earlier determined locus *QTL5.1* [30]. However, the physical location of *CQPc5.1* on the chromosome is closer than that of *QTL5.1*, so we infer that *CQPc5.1* represents a more accurate mapping of resistance to *P. capsici* in pepper.

4.3. Candidate Gene Prediction

Here, we identified five genes that are related to disease resistance in the *CQPc5.1* QTL region. We identified three genes annotated as disease-resistance protein RPP13-like; *RPP13* was a singleton NBS-LRR gene located in *CQPc5.1* on Chr05. *Capana05g000595* gene was identified as disease resistance protein RPP-8. Two genes (*Capana05g000596* and *Capana05g000597*) were annotated to be disease resistance protein RPP13-like. *RPP13* is a CC (coiled-coil)-NBS-LRR domain-containing *R* gene that controls resistance to *Peronospora parasitica* oomycete pathogen in *Arabidopsis thaliana* [53,54]. These two candidate genes encode *RPP13*-like NBS-LRR proteins and serve as potential candidates for *P. capsici* resistance in pepper. *Capana05g000598* was annotated as a probable LRR receptor-like serine/threonine-protein kinase. *Capana05g000604* was annotated to be an F-box/LRR-repeat protein. Several LRR domain proteins have been determined to participate in defense responses to infiltrating pathogens [12,55–57].

4.4. Candidate Gene qRT-PCR Analysis

Our qRT-PCR outcomes indicate that the expression patterns of three genes (*Capana05g000595*, *Capana05g000596*, and *Capana05g000597*) are up-regulated in both the resistant "PI201234" and susceptible "Early calwonder" lines after pathogen inoculation. *Capana05g000598* was down-regulated in "PI201234" with pathogen inoculation and up-regulated in "Early calwonder." We infer that *Capana05g000598* may have the part of the negative regulator of resistance to *P. capsici* in PI201234. *Capana05g000604* was up-regulated in "Early calwonder" at post-infection, while its expression level was significantly lower throughout in "PI201234". Interestingly, the expression of five genes in "PI201234" peaked 2–3 days after pathogen infection, in contrast, expression in "Early calwonder peaked at 5 day after pathogen infection." Therefore, we deduced that *Capana05g000595*, *Capana05g000596*, and *Capana05g000597* might be related to resistance to *P. capsici*. These three genes are highly associated with *CQPc5.1*, but functional validation has not been reported. Therefore, it is essential to conduct functional analysis of these genes to verify their molecular functions in *P. capsici* resistance in pepper. The result of this study would provide information for the next stage of research such as gene functional analysis, pyramiding breeding, and marker-assisted selection (MAS) as well.

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