

# Identification of Candidate Genes for Clubroot-Resistance in Brassica oleracea using QTL-Seq

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## Research Article

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# Abstract

Clubroot caused by *Plasmodiophora brassicae* is a devastating disease of cabbage (*Brassica oleracea*). To identify quantitative trait loci (QTLs) for clubroot resistance (CR) in *B. oleracea*, genomic resequencing was carried out in two sets of extreme pools that constructed from 184 F2 cloned-lines derived from cross between clubroot-resistant cabbage 'GZ87' (against race 4) and susceptible cabbage '263'. QTL-seq analyses identified one CR QTL from group I on chromosome C07 and four QTLs from group II on C04 and C07, among which three QTLs of C07 that found from group II were located within the one detected from group I. RNA-Seq and qRT-PCR were conducted in the extreme pools of group II before and after inoculation identified two potential candidate genes (*Bol037115* and *Bol042270*) from the three QTLs interval on C07, which exhibiting up-regulation after inoculation in the resistant pool but down-regulation in the susceptible pool. A functional marker 'SWU-OA' was developed from one QTL on C07, exhibiting ~95% accuracy in identifying CR in 56 F2 lines. Our study will provide valuable information on digging resistance genes against *P. brassicae* and may accelerate breeding process of *B. oleracea* with CR.

## Key Message

QTLs and potential candidate genes for clubroot resistance were identified in *Brassica oleracea*

## Introduction

Clubroot disease caused by the obligate parasite *Plasmodiophora brassicae* is a devastating disease that affects *Brassica* species worldwide including important crops like *B. oleracea*, *B. rapa* and *B. napus* (Chu et al. 2014; Dixon 2009). The pathogen usually causes gall formation on plant roots which may subsequently hamper the uptake of sufficient nutrients and water, leads to abnormal growth of plants and finally results in severe yield losses and economic damage of the crop (Devos et al. 2005). It is hard to control this disease once a field is contaminated with the pathogen since the resting spores of the soil-borne pathogen can survive in the soil for as long as 20 years (Chu et al. 2014). Cultural managements and chemical fungicides are currently the common approaches to control clubroot (Friberg et al. 2005; Ludwig-Müller et al. 2009; Peng et al. 2015), but the efficiency is unstable and the environmental damages from fungicides cannot be ignored. Therefore, developing resistant cultivars is the most effective, economical and environment friendly way to control clubroot.

Identification of clubroot resistance (CR) loci is the base of resistance breeding of *Brassica* crops. In *B. rapa*, the CR traits are reported to be controlled in a qualitative (Kuginuki et al. 1997) and quantitative manner (Suwabe et al. 2006b) depending on genotypes studied, and at least 14 race-specific loci have been identified from various European fodder turnips, such as *CRA*, *CRb*, *CRb<sup>Kato</sup>*, *CRc*, *CRd*, *CRk*, *Crr1a*, *Crr1b*, *Crr2*, *Crr3*, *Crr4*, *CrrA5*, *CRs*, *PbBa1.1*, *PbBa3.1*, *PbBa3.3*, *PbBa8.1*, *PbBrA08<sup>Banglin</sup>*, *qBrCR38-1*, *qBrCR38-2*, *Rcr1*, *Rcr2*, *Rcr3*, *Rcr4*, *Rcr5*, *Rcr8* and *Rcr9* (Chang et al. 2019; Chen et al. 2013a; Huang et al. 2019; Huang et al. 2017; Karim et al. 2020; Mehraj et al. 2020; Yu et al. 2016; Yu et al. 2017; Zhu et al. 2019). *Brassica* species containing B-genome are a useful source of genes when breeding for disease resistance (Navabi et al. 2013). A race specific CR gene (*Rcr6*) was identified in *B. nigra* (Chang et al. 2019). In *B. oleracea*, a few quantitative trait loci (QTLs) were identified, including *Pb3*, *Pb4*, *PbBo1*, *Pb-Bo (Anju) 1*, *Pb-Bo (Anju)2*, *Pb-Bo (Anju)3*, *Pb-Bo*

(*Anju*)4, *CRQTL-YC*, *CRQTL-GN\_1*, *CRQTL-GN\_2*, with limited effects on CR (Dakouri et al. 2018; Lee et al. 2015; Moriguchi et al. 1999; Nagaoka et al. 2010; Rocherieux et al. 2004; Voorrips et al. 1997) due to lack of strong resistance with the quantitative trait nature of CR in this species (Nagaoka et al. 2010). Interestingly, a CR gene (*Rcr7*) conferring strong resistance to clubroot was identified in *B. oleracea* (Dakouri et al. 2018).

In a previous study (Peng et al. 2018), several QTLs for traits such as root length and *P. brassicae* content in roots were identified from a hybrid cabbage (*B. oleracea* L. var. *capitata*) variety 'GZ87' which was resistant to *P. brassicae* race 4. However, QTLs for CR in 'GZ87' have not been identified yet. In the present study, QTL-Seq strategy (Takagi et al. 2013) was used to identify QTLs using genome resequencing in two sets of extreme pools. Potential candidate genes were identified from important QTL regions and a functional marker was further developed for molecular assisted selection. Our study will provide important significances for the breeding of clubroot-resistant cabbage.

## Materials And Methods

### Plant materials and resistance test

Two cabbage lines, 'GZ87' (with CR to *P. brassicae* race 4) and '263' (susceptible to *P. brassicae* race 4), were selected as parents to develop an F<sub>2</sub> segregating population comprising of 184 cloned-lines through tissue culture following the asexual reproduction technology (Luo et al. 2000). Vegetative plants were transplanted into 72-well plug trays after rooting and kept in a phytotron (16/8 h light/dark cycle under 25±2 °C). The inoculation of *P. brassicae* was conducted one week after transplanting, in which each plant was perfused with 5 mL *P. brassicae* (race 4) resting spores suspension (4×10<sup>7</sup> spores/mL) at the stem base using an irrigation method (Nagaoka et al. 2010; Peng et al. 2018). Fifteen plants each line with three replications were inoculated. Plants were rated at 6 weeks after inoculation according to Chen et al. (2013a) on a 0 to 4 scale, where: 0 = no clubs, 1 = less than 3 small clubs on the lateral roots, 2 = less than 3 large clubs on the lateral roots, or more small clubs, or small clubs on the taproot, 3 = large clubs on the taproot and lateral roots or many large clubs on the lateral roots, 4 = taproot rots, no lateral roots or less. Disease index (DI) was calculated according to the formula  $DI = [(0n_0 + 1n_1 + \dots + 4n_4) \times 100] / (4 \times NT)$ , where  $n_0$  to  $n_4$  are the numbers of different plants in different disease grades, and NT is the total number of plants tested. Disease incidence (DIC) was recorded as the percentage of diseased plants in the total number of inoculated plants.

### Whole genome resequencing and QTL-Seq analysis

In order to improve the reliability of QTL mapping, the F<sub>2</sub> segregating population was randomly divided into two small groups, in which group I and group II contained 110 and 74 F<sub>2</sub> lines, respectively. Each of 17/20 extreme resistant and susceptible lines were selected from group I/II. Genomic DNA was extracted from young leaves of two parents and the extreme lines following the CTAB method (Doyle 1991). A resistant (R) pool and a susceptible (S) pool were constructed within each group by mixing equal volume of DNA from the extreme resistant and susceptible lines. The two pools in group I and two parents were subjected to whole genome resequencing on an Illumina HiSeq X Ten platform in Biomarker (Beijing, China), while the two pools in group II and parents were sequenced on a NovaSeq 6000 platform in the same service provider. Reads with low-

quality bases ( $Q \leq 20$ ) or with N ratio over 10% in the pooled samples and over 1% in parental lines were removed. The high-quality reads were then mapped to the reference genome of *B. oleracea* ([http://brassicadb.org/brad/datasets/pub/BrassicaceaeGenome/Brassica\\_oleracea/Bol\\_Chromosome\\_V1.1/](http://brassicadb.org/brad/datasets/pub/BrassicaceaeGenome/Brassica_oleracea/Bol_Chromosome_V1.1/)) using BWA software (Li and Durbin 2009). The reads filtering and SNP/Indel calling were conducted according to Zhou et al. (2015).

QTL-seq analysis was performed in accordance with the method of Takagi et al. (2013). In briefly, the  $\Delta(\text{SNP-index})$  was calculated for each locus (SNP or Indel), and a sliding window analysis was applied to generate the  $\Delta(\text{SNP-index})$  curve with a window size of 100 Kb and increment of 10 Kb. Significant SNP index values (the 1% right tail) were identified as the empirical thresholds, where the threshold value was 0.34 for samples in group I and 0.35 for samples in group II.

## Transcriptome sequencing

The whole roots were collected from extreme lines in group II at 0, 4, 7 and 14 days after inoculation (DAI), with three biological replications. Roots from resistant and susceptible lines were mixed at each time point, respectively, resulting in 24 samples (R0, R4, R7, R14, S0, S4, S7 and S14, three biological replications for each) for RNA extraction and transcriptome sequencing (RNA-seq). Total RNA was extracted from these pooled root samples using TRNzol-A+ Reagent (TianGen, Beijing, China), and 24 library preparations were generated and sequenced on an Illumina HiSeq 2000™ platform in Biomarker. Clean reads were aligned to *B. oleracea* reference genome. Fragments per kilobase of transcript per million fragments mapped (FRKM) was used as the indicator of gene expression level. DESeq2 was used for identifying differentially expressed genes (Love et al. 2014). Fold Change(FC)  $\geq 2.0$  and false discovery rate ( $FDR$ )  $< 0.01$  were used as the screening criteria of differentially expressed genes (DEGs).

## Quantitative RT-PCR

Reverse transcription was conducted using the FastQuant RT Super Mix (TIAN-GEN, China). The qPCR amplification was performed by using the 2×SYBR Green qPCR Master Mix (US Everbright® Inc., Suzhou, China) on a CFX96 Touch Deep Well™ Real-Time PCR Detection System (Bio-Rad, USA) with three biological replications. Differential gene expression was calculated using the  $2^{-\Delta\Delta C_t}$  method. The *BoActin1* was used as an internal reference control. Primer sequences for all genes were available in Online Resource 1.

## Functional marker development

According to the genome sequence of interested region, a common polymorphic site between two parents and between extreme pools was developed for a molecular marker, of which the primers were 5'-TACACACGCTGATATACCAACA-3' (forward) and 5'-TACACACGCTGCCCTGGAAA-3' (reverse). The polymerase chain reaction (PCR) amplification was conducted among *B. oleracea* lines in group II. The PCR products were separated in 1.5 % agarose gels and visualized under UV light.

## Results

### Clubroot resistance of parents and extreme pools

Clubroot resistance was investigated in two parental lines and the two F2 groups across two years (Online Resource 2). In group I/II, 'GZ87' exhibited a high resistance level to *P. brassicae* (DI = 6.3/0), while '263' showed a DI value of 40/56.3. Each of 17/20 lines were selected from the 110/74 F2 vegetative lines in group I/II, presenting an average DI of 10.6/1.3 and 57.4/49.0 in the resistant and susceptible pools, respectively (Table 1). According to the DI value of the F2 population of group2, a skewed-right distribution bar graph was obtained (Fig. 1). This shows that the F2 population data we obtained were concentrated on a lower boundary, and the average DI in the population was greater than the median.

### Identification of QTLs

An average of 10.2 Gb clean data (over 20× of the reference genome) was yielded for each parental line and extreme pool in group I, with GC content of 37.7% and Q30 > 92.2%. A range of 96.7~97.3% of the clean reads were aligned to the reference genome of *B. oleracea*. A total of 1,338,555 high-quality SNPs and 3,381,999 indels were detected between the parents and between two pools (Online Resource 3). While in group II, an average of 14.2 Gb clean data was yielded for each sample, with GC content of 37.6% and Q30 > 91.26%. A range of 88.67~90.09% of the clean reads were aligned to the reference genome of *B. oleracea*, revealing 732,713 high-quality SNPs and 30,624,760 indels for the following QTL-seq analysis (Online Resource 3).

After calculating  $\Delta$ (SNP-index) of each locus and visualizing the  $\Delta$ (SNP-index) trends by sliding window method within each group, 1/4 peaks were detected in group I/II with higher values than corresponding thresholds (Table 2). One QTL (*qCRc7-1*), in an interval from 38.33 to 44.14 Mb (5.81 Mb) on chromosome C07 was identified in group I. Four QTLs, one on chromosome C04 (*qCRc4-1*, 16.92~18.79 Mb) and three on chromosome C07 (*qCRc7-2*, 38.96~39.54 Mb; *qCRc7-3*, 41.38~42.52 Mb; *qCRc7-4*, 43.56~44.15 Mb) (Table 2) were detected in group II. Comparing QTLs between two groups, the three QTLs detected in group II were all located within the locus *qCRc7-1* identified in group I (Fig. 2).

### Potential candidate genes for CR

RNA-seq was conducted in R and S pools of group II to investigate the expression of these genes. Over 6 Gb clean data was obtained from each sample (Q30 > 93%), of which 72.6~74.2% were aligned to the reference genome of *B. oleracea* (Online Resource 4). Comparing to 0 DAI, 540/500/1837 and 2656/1518/4182 DEGs were detected from the R and the S pool at 4/7/14 DAI respectively. In total, 11/16/28/16 DEGs were detected from 123/79/134/99 genes with large amounts of SNPs and indels that located within the confidence intervals of *qCRc4-1/qCRc7-2/qCRc7-3/qCRc7-4* (Table 2, Online Resource 5). Within these QTL intervals, 8 DEGs exhibiting obvious differential expression patterns between the R and the S pool were found to be located in the overlap regions on chromosome C07. The qRT-PCR revealed consistent expression patterns with RNA-seq for 6 genes among these 8 genes (Fig. 3). Among the 6 genes, *Bol037115* (FCS-like zinc finger protein, located in *qCRc7-2* with 7 SNPs and 1 indel between two pools) and *Bol042270* (plant intracellular

Ras-group-related LRR protein 8, located in *qCRc7-4* with 9 SNPs and 6 indels between two pools) were up-regulated after inoculation in the R pool but down-regulated in the S pool. The other 4 genes exhibited down-regulation after inoculation in the R pool. Therefore, *BoI037115* and *BoI042270* were considered as potential candidates for future certification.

## Functional marker for CR

According to deep comparison between two parents and between extreme pools, a PCR primer pair named 'SWU-OA' was designed within the interval of *qCRc7-4* (Fig. 2). After the amplification by SWU-OA in 54 F2 lines in group II, 28 lines that exhibited absence of the same bands (400 bp) as the resistant parent GZ87 were all susceptible to the pathogen, with DI values ranged from 20.0~75.0 (averaged in 43.2), while 26 lines that presented the 400-bp bands were found to be with DI values of 0~18.6 (averaged in 4.2), with 3 exceptions that exhibited intermediate DI values (21.4~35.0) (Fig. 4). It is indicated that the SWU-OA exhibited ~95% accuracy in identifying CR in 56 F2 lines and possibly provided a way to accelerate breeding process of *B. oleracea* with CR.

## Discussion

In the past, QTLs were mainly identified based on genetic linkage map constructed by molecular markers such as RAPD, RFLP, AFLP and SSR markers (Landry et al. 1992; Nagaoka et al. 2010; Suwabe et al. 2006a) which is a high-input but low-output approach in identifying candidate genes. With rapid development of sequencing technology in recent years, many researchers have combined bulked segregation analysis (BSA) with the whole genome and transcriptome to search for candidate genes (Dakouri et al. 2018; Zhou et al. 2020). With the complement of *B. oleracea* genome information, QTL-Seq approach (Takagi et al. 2013) combining with transcriptome sequencing made it easier to identify potential candidate genes for interested traits.

In the Fig. 1, since the mean DI in the population was greater than the median, the F2 population data we obtained were concentrated on a lower boundary and a skewed-right distribution bar graph was obtained. Different from the standard normal distribution chart, the actual data usually be biased. When the kurtosis is positive, it will form skewed-right, otherwise it will form skewed-left. In the absence of gene action, the kurtosis is negative or close to 0, while kurtosis is positive in the presence of gene interactions (Choo and Reinbergs 1982; Kotch et al. 1992; Samak et al. 2011). It is indicated that there were genes interaction in the process of constructing F2 populations between resistant and susceptible parents, studies on the amount of gene interaction are undoubtedly needed, so as to increase the efficiency of our selection and breeding programs.

As compared with that of *B. rapa*, fewer CR loci were identified from *B. oleracea* possibly due to the lack of resistant resources. The reported CR loci of *B. oleracea* were detected from chromosome C01 (*Pb3*, *PbBo1*), C02 (*Pb-Bo(Anju)1*, *Pb-Bo(Anju)2*, *CRQTL-YC*), C03 (*Pb-Bo(Anju)3*, *Pb4*), C07 (*Rcr7*, *Pb-Bo(Anju)4*) and C09 (*CRQTL-GN\_1*, *CRQTL-GN\_2*). In our study, we identified 4 QTLs for CR, of which one located on chromosome C04 and other three located adjacently on C07. No CR QTL has been detected before on C04 in *B. oleracea*, but two CR QTLs (*SCR-C4a* and *SCR-C4b*) were found from chromosome C04 in *B. napus* (Li et al. 2016). By aligning sequences of the markers in *SCR-C4a* and *SCR-C4b* to the reference genome of *B. oleracea*, the two

QTLs were aligned to 2.49–2.51 Mb and 8.06–8.10 Mb on C04 of *B. oleracea*, which were obviously distant from the interval of our *qCRc4-1* (16.92–18.79 Mb). By using the same approach, CR QTLs on chromosome C07 were compared among studies. The CR loci *PbBo(Anju)4* (Nagaoka et al. 2010) was found to locate in 37.93–39.25 Mb which was partially overlapped with our *qCRc7-2* (38.96–39.52 Mb). However, *qCRc7-2* may have a limited effect to CR since *PbBo(Anju)4* was reported to be with a very small effect ( $R^2 = 0.03$ ) and we failed in finding candidate resistance gene from this region. The other two QTLs for CR on C07, i.e. *qCRc7-3* (41.38–42.52 Mb) and *qCRc7-4* (43.56–44.15 Mb) were found to be located nearby but not overlapped with *Rcr7* (42.94–43.20 Mb) which is a major QTL in *B. oleracea* for resistance against *P. brassicae* pathotypes 3 and 5X (Dakouri et al. 2018). In addition, the most possible candidate gene for *Rcr7* (*Bo7g108760*, a TIR-NBS-LRR disease resistance gene) (Dakouri et al. 2018) was not induced by *P. brassicae* race 4 in the present study (FPKM of 0.5, 0.6, 0.4 and 0.4 in R0, R4, R7 and R14; 1.6, 0.5, 0.8 and 0.6 in S0, S4, S7 and S14, respectively). These suggest that *qCRc7-3* and *qCRc7-4* were novel loci for CR. The possible reason might be the difference on plant materials or *P. brassicae* pathotypes/races.

In a previous study, 23 QTLs for three CR-associated traits were identified from the same segregating population between ‘GZ87’ and ‘263’, including disease incidence (DIC), numbers of fibrous roots (NFR) and *P. brassicae* content in roots (PCR) (Peng et al. (2018)). Of these, 4 QTLs (*NFR.I-3*, *NFR.I-4*, *NFR.II-4* and *PCR.II-3*) located on chromosome C04 and 2 QTLs (*NFR.II-6* and *NFR.II-7*) located on chromosome C07. However, after comparison on the physic positions of these loci, none of them was overlapped with the QTLs for CR found in the current study. The closest loci between the two studies were *NFR.II-6* (47.77–48.30 Mb) and *qCRc7-4* which showed a distance of 3.62Mb on chromosome C07. This suggests that disease incidence, numbers of fibrous roots and *P. brassicae* content in roots may be not representative indicators for CR which is usually determined by disease index.

A total of 312 genes were found to locate in the three QTLs regions on C07, including 6 resistance genes (R genes) encoding TIR-NBS-LRR disease resistance proteins. However, none of these R genes presented expression difference between R and S pools in RNA-seq (Online Resource 6). Although 61 DEGs were identified from the three regions, most of them presented similar expression patterns between the two pools excepting eight genes. Among these, an FCS-like zinc finger (FLZ) domain protein (*BoI037115*) and a plant intracellular Ras-group-related LRR (PIRL) protein (*BoI042270*) exhibited over 3-fold up-regulation after inoculation in the R pool but with down-regulation in the S pool. The FLZ domain proteins are implicated in the regulation of various biotic and abiotic stresses (Chen et al. 2013b; Jamsheer K and Laxmi 2015). Members of *Arabidopsis thaliana* FLZ gene family is responsive to ABA and JA (Nietzsche et al. 2014). PIRLS encode a plant-specific class of leucine-rich repeat proteins related to Ras-interacting LRRs that take part in developmental signaling in animals and fungi (Forsthoefel et al. 2005). Some PIRL family members in rice were found to respond to different hormonal treatments, including NAA (a member of the auxin family), KT (a cytokinin), and GA (a gibberellin) (You et al. 2010). It seems that both the two candidate genes are potentially involved response to hormones which are tightly associated with host response to pathogens.

Clubroot-resistant variety is of great importance in cabbage cultivation, but the breeding practice was unsuccessful due to the lack of highly resistant sources, the quantitative nature of resistance, the difficulties in marker assisted selection (MAS) and the instability of resistance evaluation. In our study, a molecular marker (SWU-OA) that developed from the polymorphic region within *qCRc7-4* was effective in distinguishing

resistant or susceptible of F2 lines (with an accuracy of 95%), suggesting a great potential of this marker to be applied in MAS of offspring with CR. This would be helpful for lightening the labour of resistance screening in low generations and may accelerate the breeding process of *B. oleracea* with CR.

## Declarations

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## Author Contribution:

J. S, H. S. and J. M. designed and directed the experiments. F. C., H. H., Y. Z., W. H., Q. L. and X. R. performed most of experiments and analyzed the data. J. M. and F. C wrote the manuscript. J. S, H. S., F. Y. and W. Q. discussed the results and improved the manuscript.

## conflict of interest:

The authors declare that they have no conflict of interest.

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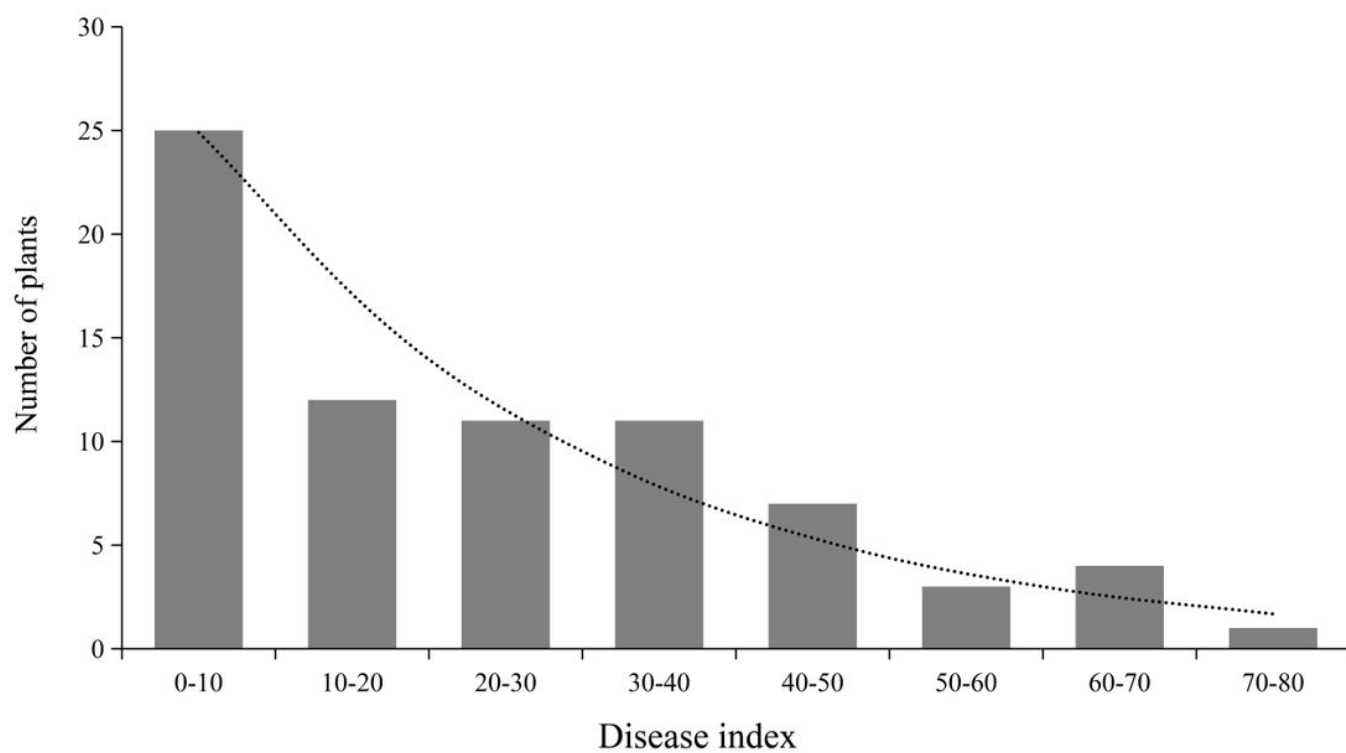
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## Tables

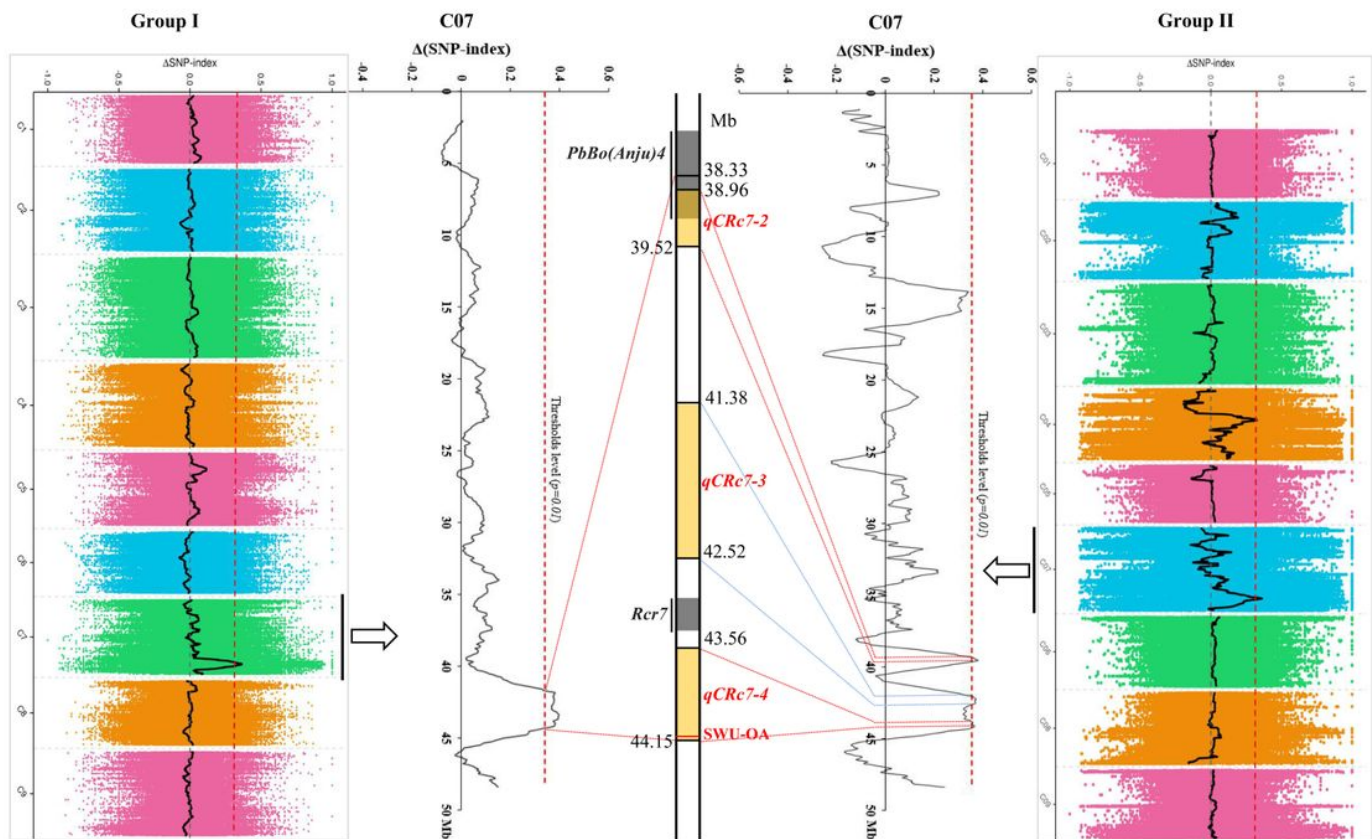
Due to technical limitations, tables are only available as a download in the Supplemental Files section.

## Figures



**Figure 1**

The DI distribution of the F2 population of group2. The abscissa in the figure is the DI value, divided by 10, the ordinate is the number of plants in each segment of the DI value, and the black dashed line is the trend line of the bar graph.



**Figure 2**

Comparison of CR QTLs identified by QTL-seq from group I (left) and group II (right). The physical map of chromosome C07 (partial) was presented in the middle, with yellow backgrounds indicating QTLs identified in this study and gray backgrounds indicating loci identified in other studies.

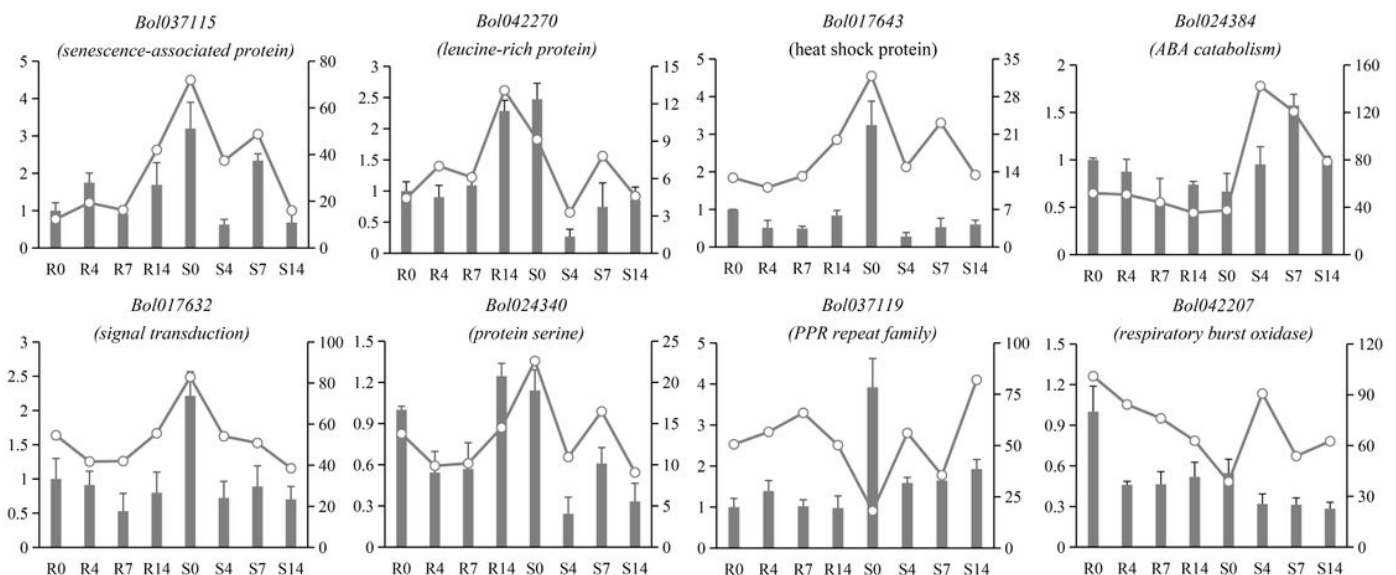


Figure 3

Comparison Expression patterns of nine DEGs locating within C07 QTL regions in resistant (R) and susceptible (S) pools before and after inoculation. The relative expression in qRT-PCR were indicated by bars with ordinate on the left; the FPKM in RNA-seq. were presented by lines with ordinate on the right side.



Figure 4

The PCR amplification of SWU-OA in two parents and F2 lines. Mismatches between the PCR products and the DI values were indicated in red.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [OnlineResource1.xlsx](#)
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- [OnlineResource6.xlsx](#)
- [Table1.xlsx](#)
- [Table2.xlsx](#)