QTL mapping for Fusarium wilt resistance based on the whole-genome resequencing and their association with functional genes in *Raphanus sativus*

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Abstract

Fusarium wilt is an important disease of radish, leading to severe decrease in yield and quality. In this study, we used 180 F_2 populations derived from a cross between radish inbred lines 'YR4' and 'YR18' to construct linkage group for the detecting quantitative trait locus (QTLs) related to *Fusarium oxysporum* resistance. Four QTLs related to *Fusarium oxysporum* resistance were detected on two linkage groups. Of the two major loci for FW resistance, $FoRsR7.1^{FOR59}$ was delimited to the 2.18-Mb physical interval on chromosome R07, with a high LOD value (5.17–12.84). Additionally, it explained 9.34%-27.97% of the phenotypic variation in three inoculation tests. The other major QTL, $FoRsR9.3^{FOR59}$, was also detected in all inoculation tests, but it had a relatively low value (3.38–4.52) and explained 6.24%-8.82% of the phenotypic variation. On the basis of the re-sequencing data for the parental lines, we identified 5 R-related genes and 13 unknown genes with sequence variations at the gene and protein levels. A semi-quantitative RT-PCR analysis revealed *Rs382940* (TIR-NBS type) and *Rs382200* (RLK type) were expressed only in 'YR4' from 0 to 6 days after the inoculation. Moreover, *Rs382950* (TIR-NBS-LRR type) was more highly expressed in 'YR4' from 3 and 6 days after the inoculation. Therefore, these three genes are likely important for the FW resistance of radish. We identified several markers based on the potential candidate genes. These gene set might be useful for breeding system to introduce the Fusarium wilt resistance loci from 'YR4' to improve increasing tolerance to *Fusarium oxysporum*.

Key Massage

Two major QTLs conferring resistance to Fusarium wilt were mapped using whole-genome resequencing. Sequence variations and gene expression level difference suggested that TIR-NBS and LRR-RLK were candidate genes. These genes were used for marker development for breeding in radish (*Raphanus sativus*).

Introduction

Radish (*Raphanus sativus*; 2n = 18), which belongs to the family *Brassicaceae*, is an economically important crop grown worldwide for its edible root and leaves. Radish is one of the most important vegetables in east-north Asia because it is used to make kimchi, which comprises traditionally fermented pickled vegetables. However, because of the repeated cultivation and excessive application of chemical fertilizers, annual radish production is adversely affected by various diseases, including soft rot, black spot, downy mildew, and Fusarium wilt (FW) (Leeman et al. 1995; Fink et al. 2005; Lingling 2018). These diseases are difficult to control and can seriously decrease radish yield and quality.

Fusarium wilt is caused by the soil-borne fungal pathogen *Fusarium oxysporum*, which seriously threatens field-grown cruciferous plants, including radish (Garibaldi et al. 2006). The pathogen propagates at soil temperatures above 24°C and can survive in soil indefinitely in the absence of living host plants (Landa et al. 2001; Pu et al. 2012; Catanzariti et al. 2017). The application of host resistance is the traditional strategy used for controlling wilt diseases. Therefore, clarifying the biological pathways

underlying disease development is conducive for generating new disease-resistant varieties. Fusarium wilt of radish, which was first reported in the USA (Snyder and Bardin 1940), develops after the pathogen penetrates the root and infects the vascular system, resulting in yellowing leaves, wilting, and stunting. Additionally, dark streaks are detectable in the xylem tissue of the lower stem and root, and the roots may decay, eventually leading to plant death and serious crop yield and economic losses (Putterill et al. 1995; Simpson and Dean 2002; Garibaldi et al. 2006; Matić et al. 2018). Because controlling FW through the application of chemical pesticides and biological control agents is difficult, breeding resistant cultivars is the most economically viable and environmentally friendly approach to managing FW.

Many mapping studies related to FW loci have been conducted for cruciferous plants. To date, at least seven FW resistance loci have been identified in *Arabidopsis thaliana* (Diener and Ausubel 2005; Cole and Diener 2013; Diener 2013; Shen and Diener 2013). In *Brassica rapa*, the candidate R genes *Bra012688* and *Bra012689*, which respectively encode TIR-NBS-LRR and NBS-LRR proteins, were identified based on analyses of differential gene expression and segregation (Shimizu et al. 2014). In an earlier study on *Brassica oleracea*, Pu et al. (2012) detected a single dominant gene, *Foc-Bo1*, mapped to linkage group 7 based on segregation and QTL analyses. This gene was cloned and confirmed to encode a TIR-NBS-LRR-type R protein (Pu et al. 2012; Shimizu et al. 2015; Pu et al. 2016). Moreover, a single dominant gene, *FOC1*, was detected on chromosome C06 based on examinations of doubled haploid and F_2 populations. The candidate gene was identified as a TIR-NBS-LRR-type R gene using gene annotations from BRAD database (Lv et al. 2013; Lv et al. 2014). Furthermore, Yu (2013) constructed a genetic map and identified eight quantitative trait loci (QTLs) in *R. sativus*, of which the major QTL, *qFW4*, was determined to be homologous to a sequence in a disease resistance-related gene cluster on *A. thaliana* chromosome 3 and 4 (Yu et al. 2013). The subsequent fine-mapping of this major locus revealed an *ORF4* sequence encoding a serine/arginine-rich protein kinase potentially related to FW resistance (Yu et al. 2019). Additionally, Yu et al. (2018) identified RGAs based on the conserved nucleotide-binding site (NBS) and S-receptor-like kinase (SRLK) domains. Developing RGA-specific primers may be useful for marker-assisted selection (Yu et al. 2018). Except for these few reports, there are no published studies regarding the identification of disease resistance-related genes or the defense mechanism underlying responses to *F. oxysporum* in radish. Thus, there is an urgent need for investigations on the genetics of FW resistance in radish.

The release of the *R. sativus* reference genome sequence comprising 46,514 genes (Mun et al. 2015), with a high (83%) genome coverage, greatly increased the efficiency in which radish genes are isolated. Additionally, the development of next-generation sequencing technology and decreases in the costs associated with sequencing have increased the popularity of plant genome re-sequencing (Liu et al. 2012; Van Dijk et al. 2014). Whole-genome sequencing provides a comprehensive overview of the genome, including the non-coding and coding regions (Meienberg et al. 2016). Moreover, it simplifies the identification of candidate genes for target traits based on the detection of sequence variations, and SNP and InDel polymorphisms following a genome alignment (Davey et al. 2011). Therefore, genome re-sequencing is very beneficial for the molecular genetic research required for plant breeding.

In this study, we analyzed ‘YR4’ and ‘YR18’ inbred radish lines, which are respectively resistant and susceptible to *F. oxysporum*, the F_2 and $F_{2:3}$ populations derived from a hybridization between ‘YR4’ and ‘YR18’. The resistant plants were screened to identify new disease resistance genes. The objectives of this study were as follows: (1) detect new FW resistance QTLs/genes in the parents; (2) construct a genetic map and develop markers linked to the novel FW resistance gene(s) by re-sequencing the genomes of the parental plants; and (3) develop markers for selecting and breeding FW-resistant radish cultivars.

Materials And Methods

Plant Materials and mapping population

The F_2 and $F_{2:3}$ mapping populations were developed by crossing inbred lines ‘YR4’ and ‘YR18’ (Provide by Neo Seed Co., Anseong, Republic of Korea), which have contrasting phenotypes regarding FW susceptibility. Specifically, ‘YR4’ is highly resistant to FW, whereas ‘YR18’ is substantially more susceptible (Ma et al, 2021). An F_1 hybrid derived from the hybridization between ‘YR4’ and ‘YR18’ was self-pollinated to produce the F_2 generation. A total of 180 F_2 plants were randomly selected to construct a genetic linkage map. Each F_2 plant was self-fertilized to produce F_3 lines, which were inoculated with fungal spores for phenotypic analyses. The fungal inoculation test was performed three times.

Pathogen inoculation

Fusarium oxysporum f. sp. *raphani* pathotypes 57, 59, and 147 obtained from the Korea Research Institute of Chemical Technology (KRICT) were used in this study. To determine the phenotypic response of the inbred lines to the pathotypes, the parental and F_1 plants were inoculated with the pathotypes in the culture room of KRICT from April 23 to May 21, and June 22 to July 18 of 2018. After selecting an appropriate pathotype, the parents and the $F_{2:3}$ population were inoculated three times (September 13 to October 12, 2018 and May 15 to June 13, 2019 in the culture room of KRICT as well as September 18 to October 24, 2018 in a glasshouse at Chungnam National University (CNU).

To prepare the inoculum, a pathogen spore suspension was grown for 2 weeks on potato dextrose agar medium, after which the density of the spore suspension was adjusted to 3×10^6 spores/mm^2 using a hemocytometer.

Disease resistance phenotypes were evaluated by inoculating the F_3 plants derived from each F_2 line. Specifically, 10 F_3 plants were grown in separate pots. They were inoculated with the spore suspension using a previously described root-dipping method (Ma et al, 2021). At two weeks after the inoculation, the disease symptoms for all plants were recorded and used to calculate the disease index (DI), which was divided into the following six grades (Fig. 1) based on symptom severity: grade 0, healthy and no symptoms in hypocotyl and leaves; grade 1, the color of the hypocotyl turns slightly brown, but symptomless in leaves; grade 2, brown hypocotyl and slightly inhibited leaf growth; grade 3: dark brown

hypocotyl, and growth delay and chlorosis in leaves; grade 4, dark brown inside of hypocotyl and completely suppressed in leaf growth; grade 5, plant death. If the score means represent one or less, it was regarded as resistant phenotype.

DNA extraction and marker development

Genomic DNA was extracted from young expanded leaves collected from greenhouse-grown plants following the CTAB method (Murray et al. 1980). A parental polymorphism survey was completed using the following markers: 662 ACMPs, EST-SSR markers in *B. rapa* (Ramchiary et al. 2011), 1,519 BRPGM SSRs (genomic-SSR markers derived from BAC end sequences in *B. rapa*), (Li et al. 2010), and 271 SSRs (EST-SSRs markers in radish), (Li et al. 2011). Additionally, 484 SNP primers were designed for a comparison with the parental whole-genome re-sequencing data.

Whole-genome re-sequencing and bioinformatics analysis

The whole genome sequencing of two parental genomes were performed paired-end by Illumina HiSeq 2500 system with 350 bp insertions. After which the generated data underwent a primary bioinformatics analysis. After a quality check, the raw reads were trimmed using “fastqc” (Andrews 2010), “jellyfish” (Marçais and Kingsford 2011), and “quake” (Kelley et al. 2010) programs. On the basis of the radish genome database, whole-genome scaffolds were assembled using SOAPdenovo (Luo et al. 2012). We performed homology and synteny analyses of the parental genomes and identified all variable sequences, including SNPs and InDels, following a mega blast analysis (Camacho et al. 2009). Then, we developed SNP and InDel primers based on the targeting sequence variation using Primer 3 program (v2.3.5) (Untergrasser et al., 2012). Those primers were used for the construction of linkage map.

Genetic map construction, comparative mapping, and QTL analysis

For genetic map construction, we used 164 lines without missing data in genotype and phenotype in common. A genetic map was constructed using JoinMap (version 4.0). Logarithm of the odds (LOD) values of 4.0–6.0 were used to assign the markers to nine linkage groups. Kosambi’s mapping function was used to convert the recombination value into map distances. The threshold for the goodness-of-fit was set to <5.0, with a recombination frequency of 0.4. The QTL analysis of FW resistance-related trait was performed using the composite interval mapping (CIM) function of Windows QTL Cartographer 2.5 (Wang et al. 2012). A LOD value was used as the minimal level for accepting the presence of a QTL based on the 1000 permutation test. To eliminate false-positive QTLs, we also performed an additional inclusive composite interval mapping (ICIM) using IciMapping 4.1 (Meng et al. 2015) to detect putative QTLs. The nomenclature of QTLs identified in our study were done as ‘*FoRsR9.1*^{FOR59}’, ‘*FoRsR9.2*^{FOR59}’ and so on, which are abbreviated as ‘*Fo*’ represents initials of ***F****usarium* ***o****xysporum*. ‘*Rs*’ stands for species ‘*Radish sativus*’, ‘*R*’ means for radish linkage group, ‘*9*’ represents chromosome number, further digit ‘*1*’ or ‘*2*’ represents number of QTL identified in the same linkage group sequentially and superscript such as ‘^{FOR59}’ represents the name of pathtype, *Fusarium oxysporum* 59, which we used in this study.

Candidate gene prediction and analysis of sequence variations in genes

The re-sequencing assembly for the parental lines were mapped to the radish reference genome (Jeong et al. 2016). All genes in the parental lines were aligned to identify sequence variations using CLC Main Workbench 7.7.1. (CLC Bio Co., Denmark). To screen the candidation genes, we considered non-synonymous amino acids and the fundamental functional annotation. Candidate genes were predicted based on orthologues gene functions with Arabidopsis at TAIR databases (https://www.arabidopsis.org/) as well as the Radish Genome Database (http://www.radish-genome.org).

RT-PCR and qRT-PCR analysis

To compare the expression level of candidate genes between two genotypes, reverse transcription (RT)-PCR and quantitative real-time (qRT)-PCR analyses were performed. We collected the leaf and root tissues from 'YR4' and 'YR18' plants at 0, 0.5, 1, 2, 4, 8, 12 hours and 1, 2, 3, 6, 12 days after the inoculation. The samples were immersed in the liquid nitrogen to be frozen as soon as harvested, and stored at -70℃. Three biological replicates of each time point were pooled used for RNA extraction by RNA Extraction kit (QIAGEN, Germany). RT-PCR was done with 2 μg total RNA was added to a 20-μl mixture to synthesize cDNA using the TOPscript™ RT DryMix kit (Enzynomic Co., Daejeon, Korea). The synthesized cDNA was diluted 10-fold, after which a 2-μl aliquot was added to each 20-μl PCR mixture as the template. The PCR primers were designed based on the radish reference genome sequence. Firstly, semi-quantitative RT-PCR was used to detect the differential expression of candidate genes in the plant root at 0, 1, 3, and 6 days after the inoculation. the PCR program was as follows: 95 °C for 3 min; 34 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The PCR amplicons were analyzed by 1% agarose gel electrophoresis. Secondly, after identified the differential expression genes, we use the qRT-PCR to confirm the expression pattern in the leaf and root of the parental lines at 0, 0.5, 1, 2, 4, 8, 12 hours and 1, 2, 3, 6, 12 days after the inoculation. qRT-PCR assay was done using a CFV96^{TM} Real-Time System (Bio-Rad Co., USA) with SYBR Green Supermix (PhilKorea, Seoul, Korea). The PCR program was as follows: 95 °C for 3 min; 39 cycles of 95 °C for 15 s, and 58 °C for 20 s. And the relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Results

Phenotypic response of radish inbred lines to three *F. oxysporum* f. sp. *raphani* pathotypes

The fungal spore inoculation test (performed twice) confirmed that inbred parental lines 'YR4' and 'YR18' were resistant and susceptible, respectively to the three *F. oxysporum* f. sp. *raphani* pathotypes (Table 1 and Additional file 2 Fig. 1). However, the symptoms on the F_1 plants inoculated with *F. oxysporum* f. sp. *raphani* pathotype 57 (*FOR57*) differed between the test replicates. In contrast, the F_1 plants inoculated with pathotype 147 (*FOR147*) were consistently susceptible to infection, but the DI was 1.0 and 3.6 in the two test replicates, implying other factors were affecting the inoculation test. Finally, in response to

pathotype 59 (*FOR59*), the F_1 plants were highly and stably resistant in the two test replicates. Thus, this pathotype was selected for subsequent analyses.

Inheritance of Fusarium wilt resistance in 'YR4'

Parental lines 'YR4' and 'YR18' were differentially affected by the inoculation with *FOR59* (mean DI of 0.1 ± 0.03, 0.3 ± 0.06 for 'YR4' and 4.6 ± 0.09, 5.0 ± 0.00 for 'YR18') (Table 1). Additionally, the F_1 plants were highly resistant to *FOR59* (mean DI of 0.0). Moreover, an analysis of 277 inoculated F_2 radish plants revealed 192 and 85 plants were resistant and susceptible to FW, respectively (Fig. 2a). The continuous distribution of DI indicated that the resistance is likely controlled by more than two genes. The DI of the $F_{2:3}$ segregating population exhibited a unimodal distribution in the 3 times independent repeat experiments for 2 years in different environments, suggesting that multiple genes were responsible for the resistance to *FOR59* (Fig. 2).

Sequencing data analysis

Sequencing data for the parental lines have generated a total of 95,021,344 and 104,493,253 raw reads generated for 'YR4' and 'YR18', respectively. Clean reads were obtained after removing adapter-containing and low-quality reads and reads with unknown bases (N > 5%), and removing the redundant reads. A total of clean reads such as 159,057,674 and 174,640,942 were secured, and those total bases length were around 13.49 Gb and 14.89 Gb. We obtained genome coverage about 25 X and 28 X for YR4 and YR18, respectively. The alignment to the reference genome indicated the read mapping rate for 'YR4' and 'YR18' was respectively 77.74% and 77.30% (Table 2).

A comparison between the 'YR4' and 'YR18' sequences revealed 161,561 SNPs and 55,674 InDels (Fig. 3). The most and fewest sequence variations were detected for chromosomes R06 and R08, which had 38,591 and 15,510 SNPs or InDels, respectively. The average number of sequence variations on the nine chromosomes was 24,249.

Development of a linkage map for *R. sativus*

To construct genetic map of radish, we surveyed the polymorphism against 2,936 markers which were previously reported in *Brassica* between YR4 and YR18. However, relatively few polymorphisms were detected with these markers. More specifically, of the 1,519 BRPGM SSRs in *B. rapa*, only 74 (4.9%) were polymorphic between the parental lines. Similarly, only 50 of 662 ACMPs (7.6%) were polymorphic. But, the markers developed in radish represented relatively high polymorphism. Of the 271 *R. sativus* EST-SSRs, only 66 (24.35%) were polymorphic between the parental lines. In the marker transferability, polymorphism was more higher within intra-species using marker developed in radishes than the analysis of result within inter-species using markers developed across Brassica species.

Additionally, 308 of 484 SNPs and InDels (63.6%) designed following the re-sequencing of 'YR4' and 'YR18' were detected as polymorphic (Additional file 1 Table S1). Overall, 498 markers were polymorphic

between the parental lines. Because we developed high-quality SNP primers based on the re-sequencing data, we did not use the 55 ACMP, BRPGM, and SSR markers for analyzing the F_2 population. Twenty markers were not linked to any chromosomes because of a highly distorted segregation or their inability to cluster within linkage groups. The radish map that was constructed had a total of 403 marker loci distributed among nine linkage groups, which were designated as chromosomes R01–R09 based on the alignment of the marker sequences to the radish genome.

The linkage map included 403 markers, covering a total distance of 1,074.23 cM, with an average distance between loci of 2.67 cM (Table 3). Additionally, R05 was the longest linkage group, spanning 182.75 cM, whereas R03 was the shortest linkage group, spanning 72.82 cM (Table 3 and Additional file 3 Fig. 2). This radish genetic map covered approximately 83% of the radish reference genome based on the comparison with physical positions of the genetic markers. Specifically, 1 cM in the genetic map corresponded to a physical length of about 320 kb.

Mapping of *F. oxysporum* resistance-related QTLs

The DI of the F_2 and $F_{2:3}$ population segregated continuously and exhibited a unimodal distribution. Among 3 times independent experiments, 2 trials were performed in controlled environment such as temperature, light, humidity, and these results were exactly replicated. But, an experiment result which had performed in glasshouse represented slightly different result, with overall the week disease symptoms. It might suggest that the resistance to *F. oxysporum* is quantitative trait instead of qualitative, and both major and minor QTLs controls resistance in *R. sativus*.

Using the radish map constructed in this study, we detected FW resistance-related QTLs via ICIM. Four different QTLs associated with FW resistance were detected in R09 (three QTLs), and R07 (one QTL) based on our phenotypic results (Table 4 and Fig. 4). These QTLs had LOD values that ranged from 3.38 to 12.84, and they explained 6.24 % to 27.97 % of the phenotypic variation. The $FoRsR7.1^{FOR59}$ and $FoRsR9.3^{FOR59}$ QTLs were detected in overlapped genomic region in three independent tests, repeatedly. Accordingly, these two loci may be important region for conferring FW resistance. Additionally, one major locus, $FoRsR7.1^{FOR59}$, explained most of the phenotypic variation (R^2) (9.34%, 22.24%, and 27.97% in three replicates during 2 years). This locus was mapped to the marker interval between R7_Rs382960 and BRPGM1176 on R07 and had a relatively high LOD value (ranging from 5.174 to 12.84).

Candidate gene identification and characterization

The $FoRsR7.1^{FOR59}$QTL, which was detected in three experiments, had a relatively high LOD value and was responsible for a considerable proportion of the phenotypic variation, suggesting it is an important major QTL related to FW resistance. We detected 238 genes in a 2.18-Mb region of chromosome R07 based on a comparison with the *R. sativus* reference genome. Furthermore, we identified homologs for 238 genes in the re-sequencing data for the two parental lines. Among these genes, 95 were associated with non-synonymous variations as well as sequence variations between the parental lines (Additional

file 1 Table S2). Interestingly, the functional annotation of these genes indicated that 5 of them were related to disease resistance, and 13 genes were unknown in functions. In addition to do that, 5 were F-box genes, 4 were zinc finger genes, 3 were MYB transcription factor genes, and 72 genes belonged to other families (e.g., genes encoding SAUR family proteins, MADS-box proteins, and ALBA RNA-binding proteins). The five disease-related genes included *Rs382950* and *Rs382940*, which encode TIR-NBS-LRR and TIR-NBS disease resistance proteins; *Rs382850* and *Rs382860*, which encode a leucine-rich repeat receptor kinase; *Rs382200*, which encodes an LRR receptor-like serine/threonine-protein kinase.

A total of 18 genes (5 R and 13 unknown genes) were confirmed using the genome re-sequencing data between the parental lines. Of these genes, we detected *Rs382940* and *Rs382200* in the genome sequence data of 'YR4', but not in 'YR18'. We designed primers (Additional file 1 Table S3) spanning a conserved region in these two genes (Additional file 5 Fig. 4a and Additional file 6 Fig. 5a). A PCR analysis using these primers demonstrated that Rs382940 and Rs382200 exist only in 'YR4', no detected with non- amplificon in 'YR18' (Additional file 5 Fig. 4b and Additional file 6 Fig. 5b). This indicates that these two genes might have important roles related to radish FW resistance.

Expression analysis and development of gene-based molecular markers

A total of 18 candidate genes (5 resistance-related genes and 13 unknown genes) were detected in the *FoRsR7.1*^{FOR59} locus. The expression patterns of these genes in root tissue of 'YR4' and 'YR18' were determined in a semi-quantitative RT-PCR assay using the 18S rRNA gene as an internal control (Additional file 1 Table S4). Both *Rs382940* and *Rs382200* were expressed exclusively in 'YR4' from 0 to 6 days after the inoculation (Fig. 5a), whereas rarely expressed in 'YR18'.

Rs382950 was highly expressed in 'YR4' than 'YR18' before inoculation. In 'YR4', expression intensity have kept strong over time after inoculation. But, in 'YR18', expression level was decreased gradually up to 3th days, and then finally it was no longer expressed in 6th days. The *Rs382850* and *Rs382860* were expressed in both lines after inoculation. In addition, *Rs382860*' expression level gradually increased after inoculation. But, in expression level not much difference were observed between two lines. There were no significant differences in the expression of the other genes. We also validated these three target genes, which shows extremely difference in expression levels by qRT-PCR.

To confirm detailed expression variation, qRT-PCR validation was performed in leaf and root tissues seperately, and investigated from the initial time after inoculation (Fig. 5b). In the *Rs382950*, the expression aspects that suddenly disapear from YR18 was reaffirmed in Day 6 condition. Two genes (*Rs382850* and Rs382860) that were expressed extremely differently between YR4 and YR18 were also confirmed to be the same as RT-PCR results. Overall, the expression patterns of YR4 and YR18 in leaf tissues were similar to those of the roots, but represented more strong expression in the root relative to leaf. These results based on the comparison of the expression level of candidate genes between two genotypes, imply these three genes might have important roles during radish responses to FW in *FoRsR7.1*^{FOR59} loci. From two major locus, *FoRsR7.1*^{FOR59} and *FoRsR9.3*^{FOR59}, six FW resistance-specific

markers were developed such as RsTNL1 (*Rs495390*), RsTNL2 (*Rs382950*), RsNL1 (*Rs382940*), RsRLK1(*Rs382200*), RsRLK2 (*Rs382860*), and RsRLK3 (*Rs380550*) based on sequence variations between the parental lines. Details regarding the primers are provided in Additional file 1 Table S5.

Using these candidate genes, the primers were designed and applied to the population. To characterize the effect of the haplotype on phenotype (disease resistance), all individuals from F_2 population were divided by haplotype, and then investigated the disease resistance with DI in each group. The individuals carrying the YR4 type allele had significantly low DI than those with the YR18 type alleles (Fig. 6, Additional file 7 Fig. 6). The disease symptom was significantly resistant in the groups having haplotype of YR4. These results demonstrated that the allele from YR4 could enhance the disease resistance.

Discussion

Breeding crops with increased disease resistance is useful for enhancing yield and quality, which will increase the economic value of crops (van Bueren et al. 2018). The use of disease-resistant cultivars is considered as one of the reliable and environmentally friendly disease control approach for sustainable agriculture (Kulwal et al. 2012). Recently, marker-assisted selection using the markers associated with the QTLs and genome information has considerably improved the selection efficiency in traditional breeding processes in various crops (Lande and Thompson 1990; Stuber et al. 1999; Collard and Mackill 2007; Varshney 2016; Jaganathan et al., 2020). However, such genomic information in radish is limited, and very few studies have been conducted on the genetics underlying radish FW resistance traits (Yu et al. 2013, 2017). To date, several radish species have been identified as resistant to various pathogens; however, the genetic basis of the resistance to the pathogen responsible for FW is largely unknown.

Fusarium wilt is one of the most critical diseases of radish, but the genetic basis of FW resistance in radish is unclear. In this study, on the basis of the DI analysis, ‘YR18’ and ‘YR4’ were confirmed as susceptible and resistant to FW, respectively. Additionally, F_1 plants were highly resistant to FW pathtype *FOR59*, indicating that the resistance is likely controlled as dominant genes in this materials. The DI of the $F_{2:3}$ population segregated continuously, and its distribution investigated in three time different inoculation tests, implying that both major and minor QTLs contribute to *R. sativus* resistance to *F. oxysporum*. The DI in the F_2 and $F_{2:3}$ population also segregated continuously, and a similar distribution was observed in different inoculation tests, indicating that the resistance is likely controlled by more than two loci, reflecting the quantitative inheritance of FW resistance in *R. sativus*. Specifically, FW resistance in *B. oleracea* and *B. rapa* were regulated by a single dominant gene (Pu et al. 2012; Lv et al. 2014; Shimizu et al. 2014). The genetic mechanism underlying FW resistance in radish differs from that in *B. oleracea* and *B. rapa* although belonging to the same cruciferous family. Among three independent trials, two trials performed in culture room displayed similar results, but that of glasshouse represent slightly different phenotype. This indicated that a controlled environment such as culture room may be one of the important factors to identify (understand) the precise phenotype.

Whole-genome re-sequencing is a powerful method for addressing fundamental evolutionary biology questions that have not been fully resolved using traditional methods (Hamilton et al. 2012; Fuentes et al. 2017; Jaqanathan et al. 2020). The rapid development of next-generation sequencing technology has greatly decreased the cost of sequencing, which is now widely used for investigating genomic structures and variations as well as for identifying candidate genes. To date, many research groups have sequenced different radish lines and reported the construction of *R. sativus* reference genome sequences (Kitashiba et al. 2014; Mitsui et al. 2015; Jeong et al. 2016). The latest genome sequence released by Jeong et al. (2016) has a high genome coverage (83%) and includes 46,514 predicted genes. In the current study, we re-sequenced the parental lines and assembled a reference genome using the Rs1.0 version of the radish genome. The alignment of the parental sequences enabled us to predict 218,237 SNPs and InDels that were further used to construct linkage groups. A high proportion of polymorphic markers (54.07%) was mapped to the radish linkage groups. Additionally, the re-sequencing data for the parental lines simplified the prediction of candidate genes in the QTL regions. In the present study, on the basis of the linkage map, we identified four QTLs (two robust major and two minor) (Fig. 4). Of the two major loci, $FoRsR7.1^{FOR59}$ was mapped to chromosome R07, with a physical length of 2.18 Mb and an LOD value between 5.17 and 12.84; this QTL explained 9.34–27.97% of the phenotypic variation in three consecutive inoculation tests. The other major QTL, $FoRsR9.3^{FOR5}$, had a relatively low LOD value (3.38–4.52) and explained 6.24–8.82% of the phenotypic variation. Thus, we speculated that $FoRsR7.1^{FOR59}$ is an important locus associated with FW resistance. We revealed that $FoRsR7.1^{FOR59}$ includes approximately 238 genes, which should be further investigated to identify candidate genes for FW. Furthermore, these genes were narrowed downed to 95 genes based on DNA sequence variations (re-sequencing of parental lines) and the associated amino acid changes.

In previous studies, Yu (2013) identified eight QTLs related to FW resistance in radish. On the basis of fine-mapping, *ORF4*, which encodes a serine/arginine-rich protein kinase family member, was suggested as a potential candidate gene for FW resistance. Interestingly, a major QTL detected in the current study, $FoRsR9.3^{FOR59}$, was associated with the previous identified QTL (*qFW7*) (Yu et al. 2013). The similarity of the locus might be related to the similarity in the genetic background of the plant materials analyzed in the two studies. Although it was not the most notedly loci in both studies, the results mutually detected in the two studies performed independently of each other might suggest that this locus may have a broad regulatory role affecting FW resistance.

Accordingly, $FoRsR7.1^{FOR59}$ on R07 is a novel locus for the resistance to pathotype *FOR59*, and $FoRsR9.3^{FOR59}$ on R09 overlap the locus previously identified by Yu et al (2013) (Fig. 7). So far, in plants more than 100 R genes have been identified and cloned, and most of them are nucleotide-binding site leucine-rich repeat (NBS-LRR) genes, receptor-like kinase (RLK) genes, and receptor-like transmembrane protein (RLP) genes (Voorrips et al. 1997; Saito et al. 2006; Kato et al. 2013; Lv et al. 2014; Shimizu et al. 2015; Liu et al. 2019). Research regarding *B. oleracea* and *B. rapa* indicated the R genes responsible for FW resistance are TIR-NBS-LRR-type genes (Lv et al. 2014; Shimizu et al. 2014, 2015).

In the present study, five R genes located in the major QTL *FoRsR7.1*^{FOR59}on R07 had variations in DNA sequences, which resulted in diverse amino acid sequences. Both *Rs382850* and *Rs382860* are homologous to *AT1G73080*, which encodes a leucine-rich repeat receptor kinase (LRR-RLK) that serves as a receptor for AtPep1 to enhance the innate immune response to pathogen attacks in *A. thaliana* (Ryan et al 2007; Yamada et al. 2016; Jing et al. 2020). The *Rs382950* gene belongs to the TIR-NBS-LRR gene family and is homologous to *AT5G46450* and *AT1G72840* in *A. thaliana*. Similarly, *Rs382940* encodes a TIR-NBS protein and is related to *AT4G09420*, whereas *Rs382200* is an ortholog of *A. thaliana AT1G74360-like*. This *AT4G09420* gene encoding NB-LRR protein was reported to be induced as a basal defense responses, which can function together in the recognition of pathogenes (Nandety et al. 2013), and implicated in response to *Fusarium* infection (Le et al. 2014; Schumann et al. 2017). The *A. thaliana* gene *AT1G74360* encodes a leucine-rich repeat receptor-like serine/threonine protein kinase (LRR-RLK) involved in immune responses initiated by nematodes (Mendy et al., 2017), and involved in the banana resistance against Fusarium, cause of sensing and perception of the pathogen signal (Niu et al. 2018). Although these potential candidate genes selected from the QTL regions based on the presumably interesting functions reported in previous studies, further advanced research should be implemented to identified the roles and elucidate the mechanism of resistance.

In conclusion, using the F_2 population, we identified and mapped four QTLs associated with FW resistance on two chromosomes. Of these QTLs, *FoRsR7.1*^{FOR59} and *FoRsR9.3*^{FOR59} were considered to be an important major QTLs related to radish FW resistance. Six gene-based markers used in combination may considerably enhance marker-assisted selection for breeding new radish lines with increased resistance to FW.

Declarations

Acknowledgments

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Author's contributions

SRC and YPL and designed the study. YM carried out experiments, generated data. SRC, YM analyzed all data, and drafted manuscript. SSC participated in data analysis, writing and editing of the manuscript. LL did marker survey and genotyping. LL, SK, GJC were participated in phenotype evaluations. YPL provided plant materials, conceived the study, and finalized the manuscript. SRC conceived and designed the study, participated as a director, and modified the manuscript. All authors read and approved the final manuscript. All the authors declare that they have no conflicts of interest.

Compliance with ethical standards

Conflict of interest

The authors declare no conflict of interest.

Ethical standards The experiments comply with the laws of the USA, the country in which the study was performed, and the ethical standards of the respective university and employers of the authors.

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Tables

Due to technical limitations, Tables 1-4 are only available as a download in the supplemental files section

Supplemental Data List

Additional Files Supplementary information

Additional file 1: Table S1: The marker information and polymorphic survey result.

Additional file 1: Table S2: List of the genes in the *FoRsR7.1*^{FOR59}region that have sequence variations between the parental lines.

Additional file 1: Table S3: List of primers used for check two genes in parental lines.

Additional file 1: Table S4: List of primers for checking the expression of 6 R genes and 13 unknown genes.

Additional file 1: Table S5: List of the primers used for screening populations for FW resistance.

Additional file 2 Fig. 1 Symptoms of plants infected with *Fusarium oxysporum* f. sp. *raphani* pathotypes 59, 57, and 147 at 25 °C and 60% relative humidity, with a 12-h light condition cycle in a culture room at the Korea Research Institute of Chemical Technology (KRICT).

Additional file 3 Fig. 2 Genetic map of radish showing nine linkage groups.

Additional file 4 Fig. 3 QTLs for Fusarium wilt resistant traits to *Fusarium oxysporum* f. sp. *raphani* pathotype 59 in $F_{2:3}$ population of *R. sativus* by two different software, WinQTL 2.5 and IciMapping 4.1. (a), (b) represented the results of 9 chromosome scane overall radish genome. (c), (d) represented the QTL regions identified on R7 in two different analysis methods by WinQTL 2.5 based on Composite

Interval Mappin (CIM) and Inclusive Composite Interval Mapping (ICIM). Tree different replicates were performed during 2 years in culture room and glasshouse.

Additional file 5 Fig. 4 PCR amplification profiles of *Rs382940* in 'YR4', 'YR18', and F_1 plants.

Additional file 6 Fig. 5 PCR amplification profiles of *Rs382200* in 'YR4', 'YR18', and F_1 plants.

Additional file 7 Fig. 6 Box plots of disease index (DI) variation at different haplotypes of alleles. The central line of box means median, and box limits are the upper and lower quartiles,. The significance of difference was analysed with T-Test and one way ANOVA and in all haplotypes significant difference were observed ($P<0.001$).

Figures

Figure 1

Disease symptoms of radish plants infected with Fusarium oxysporum f. sp. raphani pathotype 59. The following scale was used to evaluate the disease symptoms: grade 0, healthy and no symptoms in hypocotyl and leaves; grade 1, the color of the hypocotyl turns slightly brown, but symptomless in leaves; grade 2, brown hypocotyl and slightly inhibited leaf growth; grade 3: dark brown hypocotyl, and growth

delay and chlorosis in leaves; grade 4, dark brown inside of hypocotyl and completely suppressed in leaf growth; grade 5, plant death.

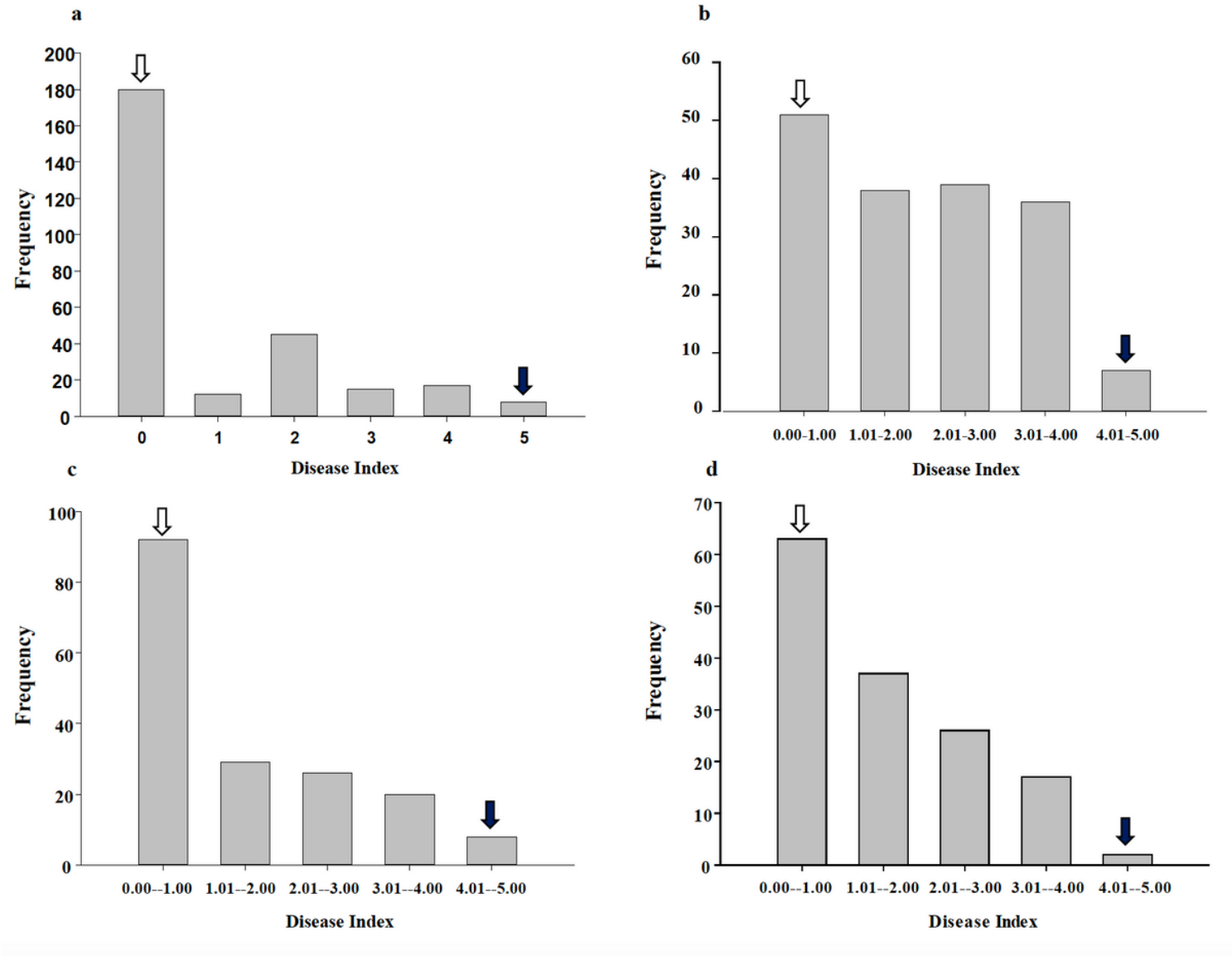

Figure 2

Frequency distribution of disease symptoms investigated for Fusarium wilt. (a) F2 population in 2018 (n = 277), (b) F2:3 population infected at a glasshouse of CNU in 2018 (n = 171), (c) and (d) F2:3 population at culture room in 2018 (n =175) and 2019 (n =145). White and black arrows represent the value of resistant line, ‘YR4’ and susceptible line, ‘YR18’.

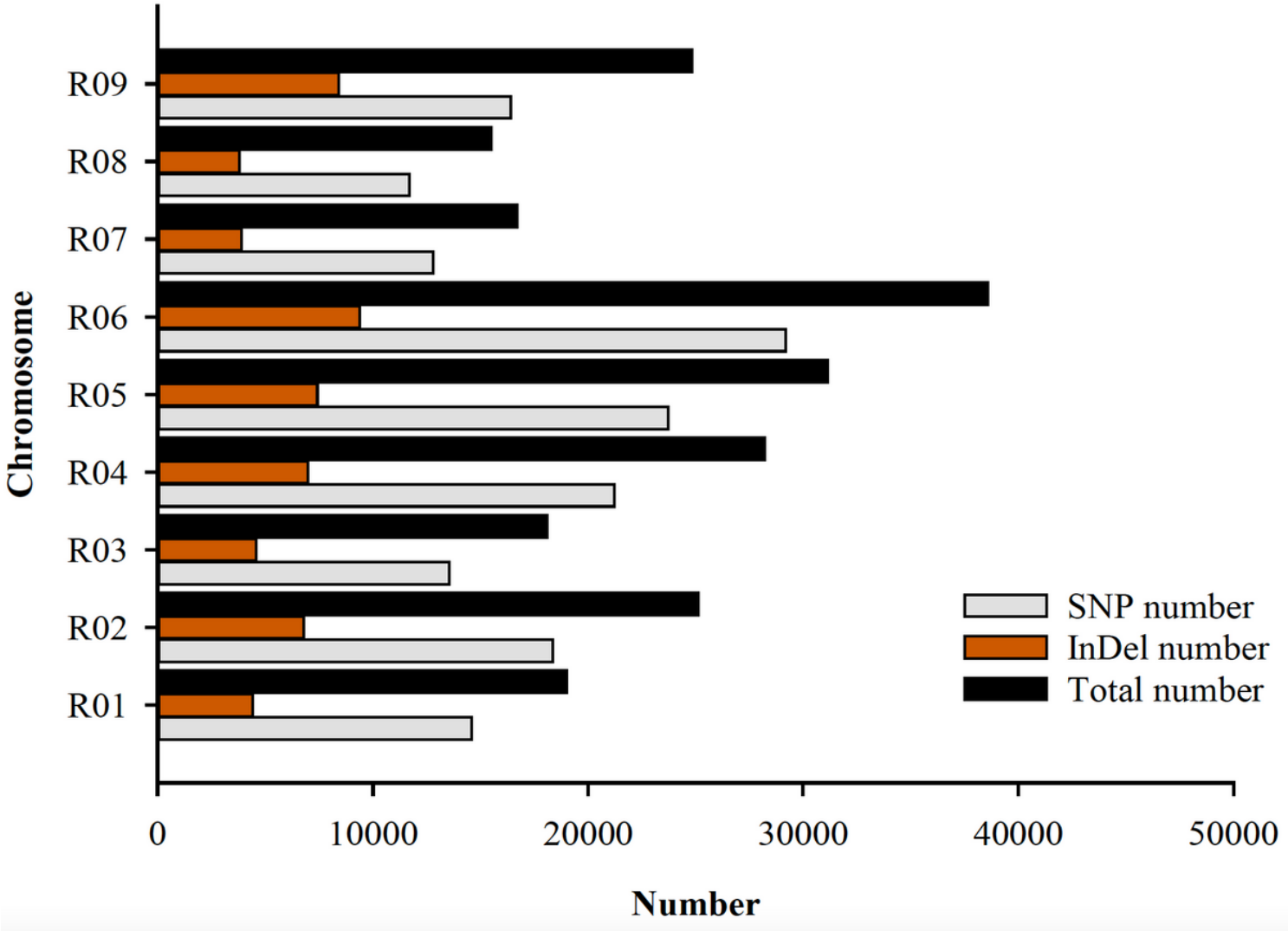

Figure 3

Chromosomal distribution of the SNPs and InDels between ‘YR4’ and ‘YR18’ plant genomes.

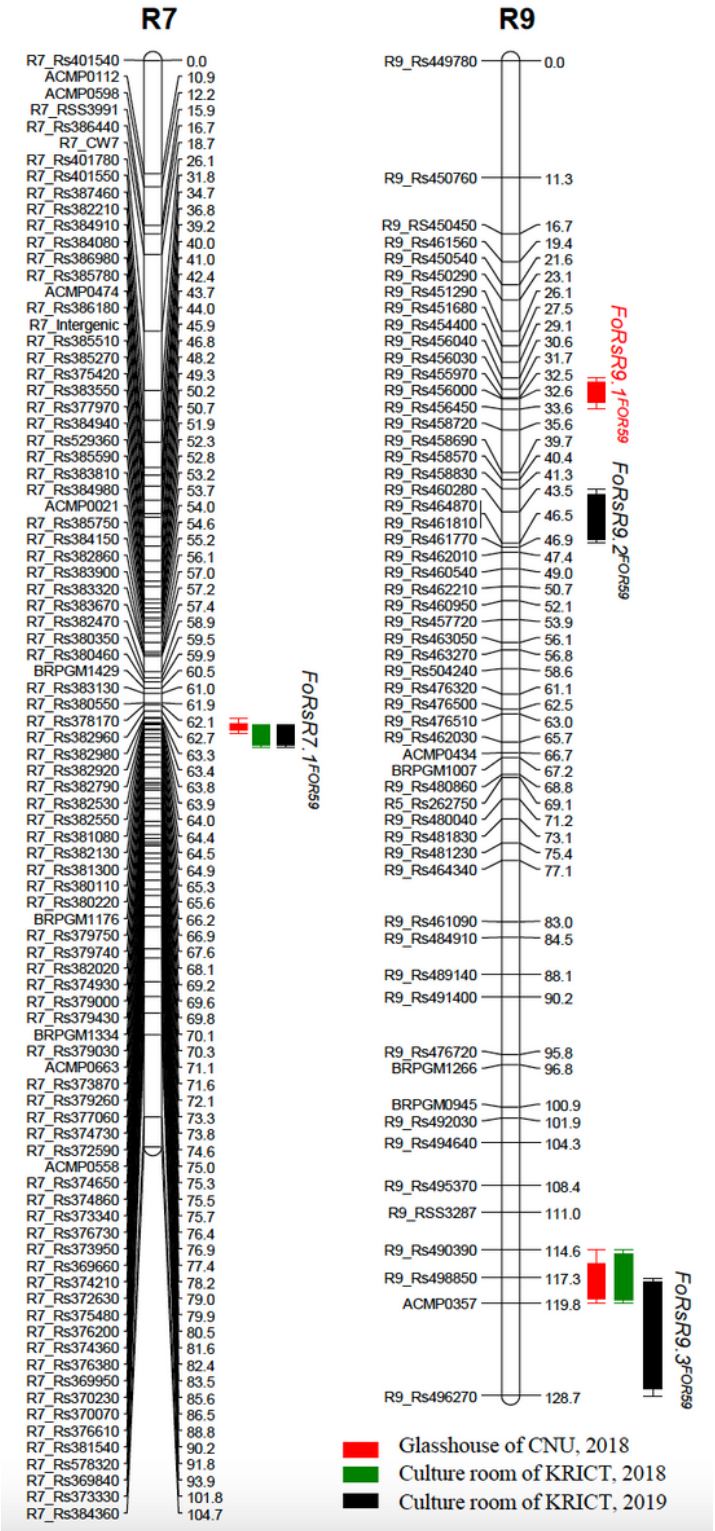

Figure 4

Genetic linkage map and distribution of QTLs for Fusarium wilt resistant traits to Fusarium oxysporum f. sp. raphani pathotype 59 in F2:3 population of R. sativus. The markers are showen to the right side of linkage group, and genetic distances are represented to the right side of linkage group as centi-morgans (cM). QTLs identified were indicated by abbreviations of trait names in the right side of linkage group,

and summarized in Table 4. The rectangular bars of red, green, black color shown the QTL region, detected by IciMapping 4.1 in repeated experiments, respectively.

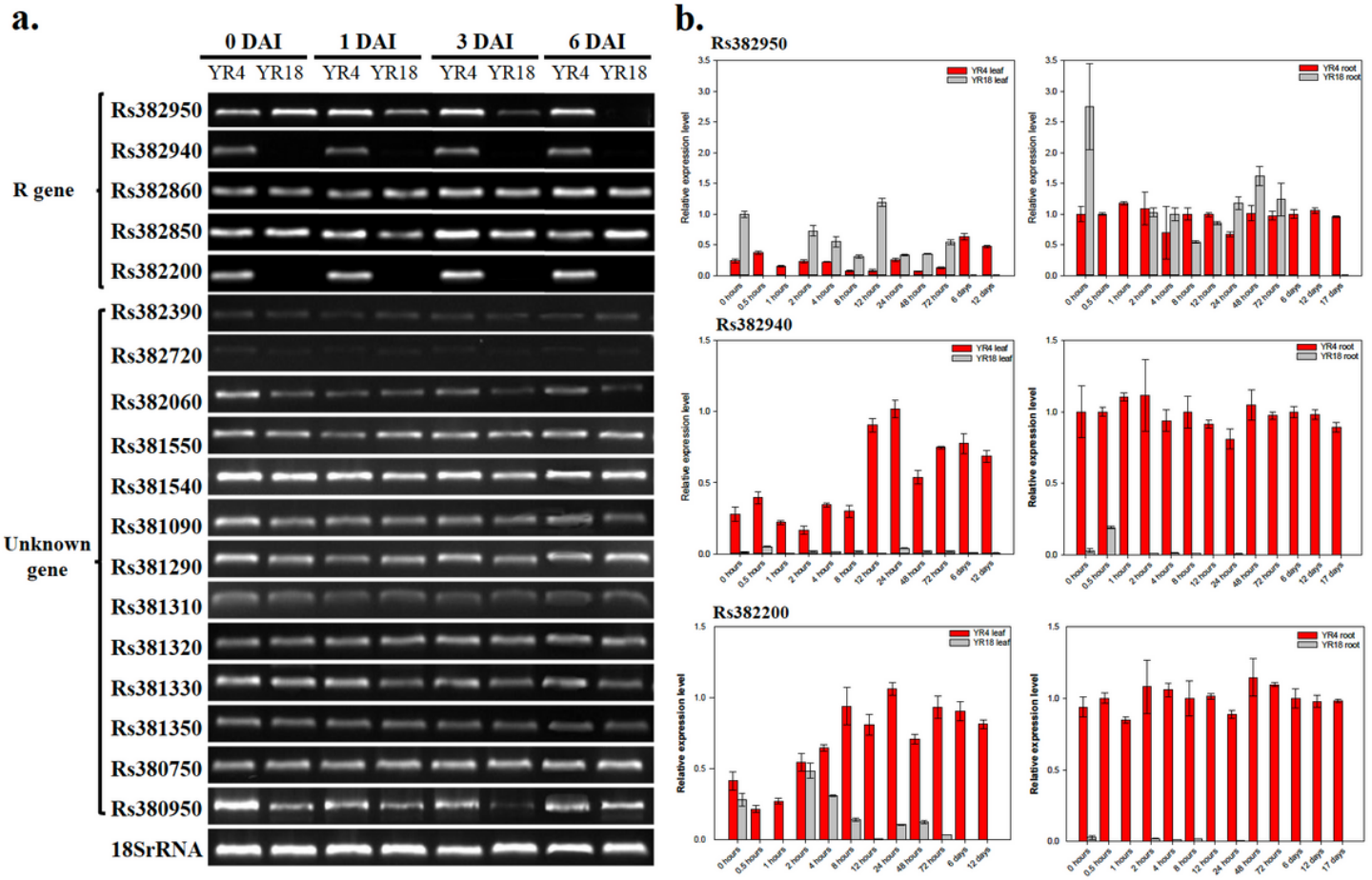

Figure 5

Expression of 5 R genes and 13 unknown genes between resistant line ‘YR4’ and susceptible line ‘YR18’ by RT-PCR (a), and qRT-PCR (b). Roots were collected from 1, 3, 6 days after inoculation (DAI) with Fusarium oxysporum f. sp. raphani pathotype 59 for RNA extraction for RT-PCR. The sample, 0 DAI, represented non-inoculated roots. The samples collected from leaf and root tissues as time course were used for qRT-PCR; 0.5, 1, 2, 4, 8, 12 hours, and 1, 2, 3, 6, 12days.

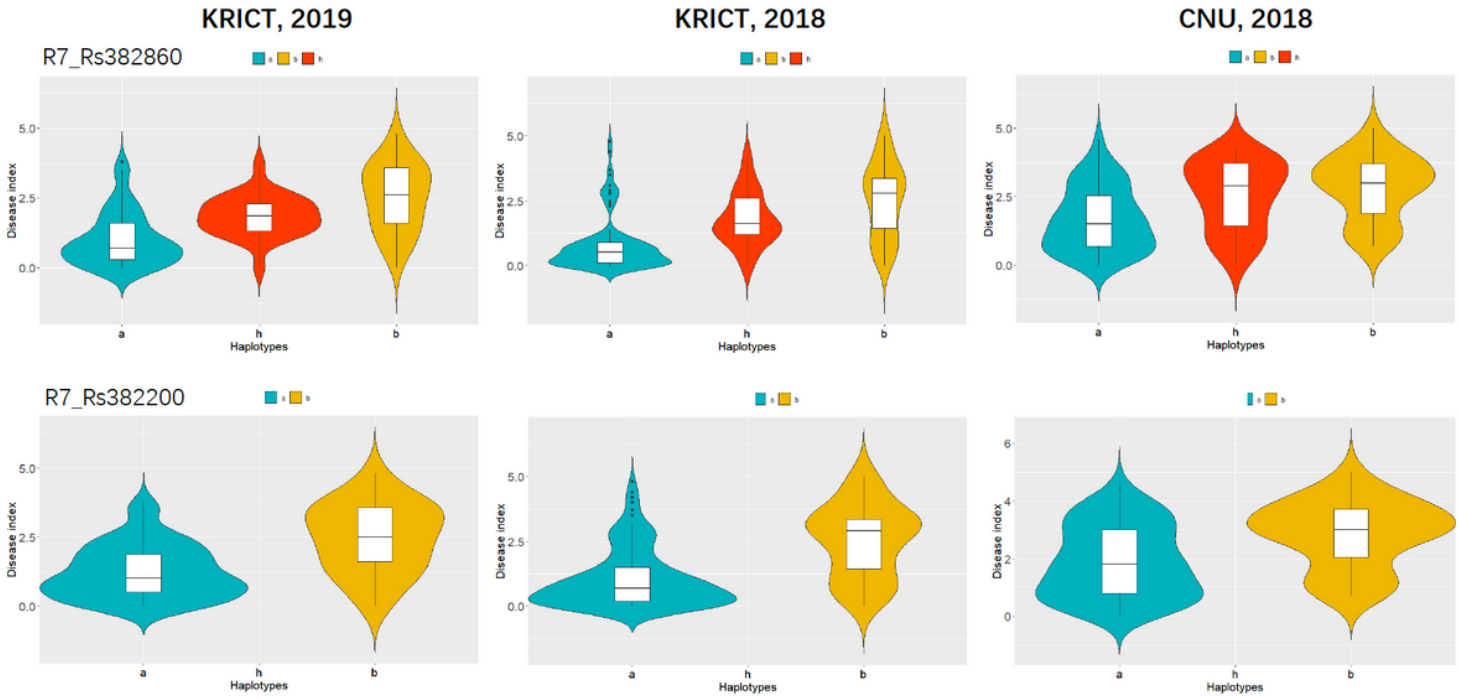

Figure 6

Box plots of disease index (DI) variation at different haplotypes of alleles. The central line of box means median, and box limits are the upper and lower quartiles,. The significance of difference was analysed with one way ANOVA.

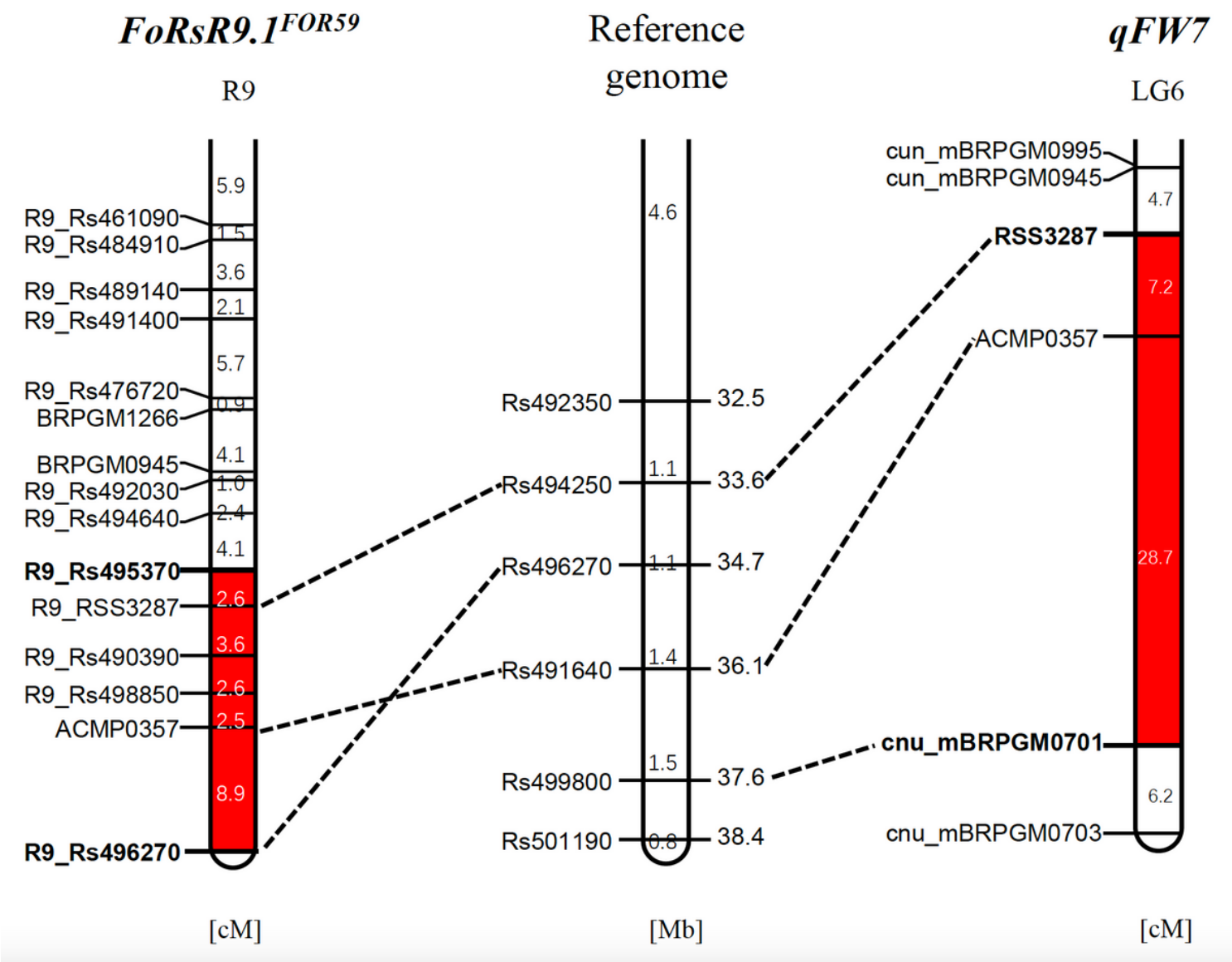

Figure 7

Synteny relationship of the two Fusarium wilt resistance loci, FoRsR7.1FOR59 identified in this study and qFW7 which is previously reported (Yu et al. 2013). The center represented reference genome. In the genetic map, the QTL area was indicated in red color. The anchor markers which used in two studies as a common, and markers with similar physical location in the reference genome were connected by broken lines.

Supplementary Files

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

- Table1..xlsx
- Table2..xlsx
- Table3..xlsx

- Table4..xlsx
- Additionalfile2Fig.1.pdf
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- Additionalfile4Fig.3.pdf
- Additionalfile5Fig.4.pdf
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- AdditionalfilesSupplementaryTable3.xlsx
- AdditionalfilesSupplementaryTable4.xlsx
- AdditionalfilesSupplementaryTable5.xlsx