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

Key message Two major QTL associated with resistance to Fusarium wilt (FW) were identified using whole-genome resequencing. Sequence variations and gene expression level differences suggest that TIR-NBS and LRR-RLK are candidate genes associated with FW-resistance.

Abstract Fusarium wilt (FW) caused by Fusarium oxysporum f. sp. raphani is an important disease in radish, leading to severe decrease in yield and quality. YR4 as a novel genetic source to resistant to FW was confirmed through screening with five pathogen isolates. We have generated F2 and F2:3 populations segregated with FW resistance using YR4 and YR18 inbred lines. The disease symptom was evaluated in F2:3 population (n = 180) in three independent studies over two years. We identified 4 QTL including the two major QTL (FoRsR7.159A and FoRsR9.359A). FoRsR7.159A and FoRsR9.359A were detected in three replicated experiments. FoRsR7.159A was delimited to the 2.18-Mb physical interval on chromosome R07, with a high LOD value (5.17–12.84) and explained phenotypic variation (9.34%–27.97%). The FoRsR9.359A represented relatively low LOD value (3.38–4.52) and explained phenotypic variation (6.24%–8.82%). On the basis of the re-sequencing data for the parental lines, we identified five putative resistance-related genes and 13 unknown genes with sequence variations at the gene and protein levels. A semi-quantitative RT-PCR analysis revealed that Rs382940 (TIR-NBS) and Rs382200 (RLK) were expressed only in ‘YR4’ from 0 to 6 days after the inoculation. Moreover, Rs382950 (TIR-NBS-LRR) was more highly expressed in ‘YR4’ from 3 to 6 days after the inoculation. These three genes might be important for FW-resistance in radish. We identified several markers based on these potential candidate genes. The marker set should be useful for breeding system to introduce the FW resistance loci from ‘YR4’ to improve tolerance to FW.

Introduction

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

Fusarium wilt is a major radish disease, caused by the soil-borne fungal pathogen Fusarium oxysporum, which severely damages diverse agricultural crop plants such as tomato, watermelon, banana, cotton, cabbage, Chinese cabbage, radish etc. by different formae speciales (f.sp.) (Garibaldi et al. 2006; Husaini et al. 2018; Dean et al., 2012; Branham et al., 2020).

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). 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; Gari-baldi et al. 2006; Matić et al. 2018). Controlling FW through the application of chemical pesticides and biological control agents is difficult. On the other hand, breeding for resistant cultivars is the most economically viable and environmentally friendly approach to managing FW. The application of host resistance is the traditional and effective strategy used for controlling wilt diseases for sustainable agriculture. Therefore, understanding the biological pathways involved disease development and identifying the genetic factors are helpful for developing new disease-resistant varieties.

Many genetic studies have been conducted in cruciferous plants to identify genetic factors related to FW-resistance loci. 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 an earlier study on *Brassica oleracea*, Pu et al. (2012) detected a single dominant gene in *FocBo1* locus, mapped to linkage group 7 based on segregation and QTL analyses against *F. oxysporum* f. sp. *conglutinans*. Lv et al. (2013) identified *FOC1* controlling as a single gene, on chromosome C06 based on examinations of doubled haploid and F2 populations. And the candidate gene, *Bol037156*, was identified by transcriptome analysis as a Toll interleukin receptor nucleotide binding site-leucine rich repeat (termed TIR-NBS-LRR) type resistance gene (*R*-gene) based on gene annotations from BRAD database (Lv et al. 2014). And the genes located in candidate region represented the syntenies to the Arabidopsis chromosome 4. In *Brassica rapa*, the two candidate *R*-genes, *Bra012688* and *Bra012689*, which encode TIR-NBS-LRR and NBS-LRR proteins, were identified based on analyses of differential gene expression (Shimizu et al. 2014). In *R. sativus*, Yu et al. (2013) identified and reported eight quantitative trait loci (QTL) against *F. oxysporum* f. sp. *raphani* ‘HN’ and ‘JHW’. The qFW4, located in LG3 (Chromosome R5), was constantly detected in three independent biological experiments in two consecutive-years. Thus, it was considered as a major QTL and was named ‘*Fwr1*’ (Fusarium wilt resistance loci in radish as a first). The syntenic analysis of ‘*Fwr1*’ region is homologous to disease resistance-related gene cluster in *A. thaliana* chromosome 3 (Yu et al. 2013). In contrast for in crops like cabbage and Chinese cabbage, comparative syntenic analysis for resistant loci, shown homologous to disease resistance-region in *A. thaliana* chromosome 4 (Pu et al. 2012; Shimizu et al. 2014, 2015). It suggests that the resistance genetic factors in radish vary even among the same cruciferous family species (cabbage and Chinese cabbage).

Further, ‘*Fwr1*’ QTL regions were narrow down to 139.8 kb through fine mapping approach using 2,627 large-scale *F2* population and identified an *ORF4*, encoding a serine/arginine-rich protein kinase (Yu et al., 2020). In addition, Yu et al. (2018) identified resistance gene analogs (RGAs) based on the conserved nucleotide-binding site (NBS) and S-receptor-like kinase (SRLK) domains. Developing RGA-specific primers might be useful for marker-assisted selection (Yu et al. 2018). Except for these few reports, to our knowledge there are no published studies regarding the identification of disease resistance-related genes or the defense mechanism involved in 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 decrease in the cost have increased the popularity of plant genome resequencing (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 single nucleotide polymorphisms (SNPs) and insertions and deletions (InDels) 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 used radish inbred lines ‘*YR4*’ and ‘*YR18*’, which are resistant and susceptible to *F. oxysporum* f. sp. *raphani* 59A. Both of these lines showed susceptible phenotype in response to *F. oxysporum* f. sp. *raphani* ‘*HN*’ and ‘*JHW*’ isolates which were previously used by Yu et al. (2013, 2020). It suggests that the genetic loci controlling resistance trait in ‘*YR4*’ to *Fusarium wilt* may have different loci from the previous studies of Yu et al. (2013, 2020). Therefore, the objectives of this study are as follows: (1) To detect new FW resistance QTL/genes in the parental inbred line ‘*YR4*’ against pathotype ‘*59A*’; (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 identifying FW-resistant radish cultivars.

**Materials and methods**

**Plant materials and mapping population**

The *F2* and *F2:3* 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 F₁ hybrid derived from the hybridization between ‘YR4’ and ‘YR18’ was self-pollinated to produce the F₂ generation. Each F₂ plant was self-fertilized to produce F₂:₃ population. We have generated F₂:₃ population consist of 180 lines/families. Each family was tested of 10 individual plants in triplicates (i.e., total 30 F₂:₃ plants per family/line). Finally, 180 F₂:₃ families and two parental lines were evaluated for Fusarium disease phenotype using a randomized block design with three replicates over two years. To evaluate the resistance characteristics of radish to Fusarium oxysporum infection, we used the ten individual plants of ‘YR18’ and ‘Backchun’ (Lee et al. 2020) as susceptible control (susceptibility) to Fusarium oxysporum infection. Five individuals of each of these plants were used as mock-inoculated (infected with water) as negative control in all experiments.

Pathogen inoculation

To determine the phenotypic response of the inbred lines to the pathotypes, the parental lines and F₁ plants were inoculated with the five different Fusarium oxysporum f. sp. raphani pathotypes. Fusarium oxysporum f. sp. raphani pathotypes ‘57A’, ‘59A’, and ‘147A’ obtained from the Korea Research Institute of Chemical Technology (KRICT) reported by Kim et al. (2017) and Lee et al. (2020) were used in this study including the pathotype ‘HN’ and ‘JHW’ used in previous reports by Yu et al. (2013, 2020). The evaluation of disease symptom was performed in the culture room of KRICT from April 23 to May 21, and June 22 to July 18 of 2018. Among five different pathotypes, depending on the phenotypic response, F. oxysporum f. sp. raphani ‘59A’ was selected for further study in F₂ and F₂:₃ population to identify genomic regions and genetic factor associated to resistance of ‘YR4’.

To determine disease resistance phenotype (trait) of each F₂:₃ families, and excluding false positive results based on the each environmental condition, we evaluated disease symptom/disease resistance phenotype of 180 F₂:₃ families/ population (i.e., total 30 F₂:₃ plants per family/line) and two parental lines with three independent studies over two years (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).

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 × 10⁶ spores/mm² using a hemocytometer. They were inoculated with the spore suspension using a previously described root-dipping method (Ma et al. 2021). Ten-day-seedling were inoculated into the spore suspension of Fusarium oxysporum prepared by the methods previously described, and then transplanted into a separate pots (5 cm × 5 cm × 5 cm), individually. After inoculation, all individual plants were randomly re-planting into a new separate pots and grown under the identical condition such as soil autoclaved, light condition (12 h/12 h), 60% humidity, irrigation, and 25 °C temperature within controlled environments. In each F₂:₃ families, 10 individual plants were used for inoculation, and the average values of F₂:₃ families were used for QTL analysis, respectively. Due to poor germination and irregular conditions of seedling growth, some lines were not sufficient to obtain phenotypic data. Additionally, we also used F₂ population to observe the natural segregation pattern of resistance trait (n = 277).

At two weeks after the inoculation, the disease symptoms for all plants were recorded and used to calculate the disease severity, 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 and Thompson 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”, “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) (Untergasser 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. SNP markers were generated from ‘YR4’ and ‘YR18’ genome re-sequencing data, and the SSR markers which were previously reported in Brassica (Ramchary et al. 2011; Li et al. 2010; Shirasawa et al. 2011) were used for the map construction and QTL analysis (Additional file 1 Table S1). The QTL analysis of FW resistance-related trait was performed using Windows QTL Cartographer 2.5 as a composite interval mapping (CIM) method (Wang et al. 2012). For the CIM analysis, we used model 6 (standard model) which automatically select the control markers by using additional parameters as follows; control marker default values 5, window size was 10 cM with forward and backward regression method. And for precise results, we decreased the walk speed (cM) as 1. A LOD value was used as the minimal level for accepting the presence of a QTL based on the 1000 permutation test with significance level of 0.05 (Churchill and Doerge 1994; Van Ooijen 1999). To eliminate false-positive QTL, we also performed an additional inclusive composite interval mapping (ICIM) using IciMapping 4.1 (Meng et al. 2015) to detect putative QTL. ICIM was performed using the default parameters as follows; Step = 1 cM; phenotype on marker variables (PIN) = 0.005. The LOD threshold was based on the 1000 permutation at a significance level of 0.05. The nomenclature of QTL identified in our study were done as ‘FoRsR9.139A’, ‘FoRsR9.239A’ and so on, which are abbreviated as ‘Fo’ represents initials of Fusarium oxysporum. ‘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 ‘39A’ represents the name of pathotype, Fusarium oxysporum 59A, 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 h 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 °C. 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 tenfold, 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 h and 1, 2, 3, 6, 12 days after the inoculation. qRT-PCR assay was done using a CFV96™ 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−ΔΔCt method (Livak and Schmittgen 2001).

Results

Phenotypic response of radish inbred lines to five 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 (59A, 57A, 147A). But, both of them were clearly susceptible to the pathotypes (HN, JHW) (Table 1 and Additional file 2 Fig. 1). However, the symptoms on the F1 plants inoculated with F. oxysporum f. sp. raphani pathotype 57A (57A) differed between the test replicates. In contrast, the F1 plants inoculated with pathotype 147A (147A) 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 59A (59A), the F1 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 59A (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 F1 plants were highly resistant to 59A (mean DI of 0.0). Moreover, an analysis of 277 inoculated F2 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

| Line (n = 10)a | F. oxysporum f. sp. raphani pathotype | 59A | 57A | 147A | HN, JHWd |
|----------------|------------------------------------|-----|-----|------|----------|
|                | DIb phenotypec                       | DI phenotype | DI phenotype | DI phenotype | DI phenotype |
| F1 (YR 4 × YR 18) | 0.0 ± 0.00 R | 0.4 ± 0.10 R | 1.0 ± 0.31 S | 4.6 ± 0.14 S |          |
| YR 4           | 0.3 ± 0.06 R | 0.5 ± 0.08 R | 0.9 ± 0.15 R | 5.0 ± 0.00 S |          |
| YR 18          | 5.0 ± 0.00 S | 5.0 ± 0.00 S | 5.0 ± 0.00 S | 5.0 ± 0.00 S |          |
| F1 (YR 4 × YR 18) | 0.0 ± 0.00 R | 4.2 ± 0.15 S | 3.6 ± 0.20 S | 4.5 ± 0.11 S |          |

aNumber of plants in experiments as repeat in each trials
bDI: Disease index
cR: resistance; S: susceptible
dThese two pathotypes, HN and JHW, were used in previous study by Yu et al. (2013)
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 59A (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 $25\times$ and $28\times$ 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

![Fig. 2](image-url)
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 F2 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 *Fusarium oxysporum* resistance-related QTL**

The DI of the F2 and F2:3 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. Using the radish map constructed in this study, we detected FW resistance-related QTL via Inclusive Composite Interval Mapping (ICIM). Four different QTL associated with FW resistance were detected in R09 (three QTL), and R07 (one QTL) based on our phenotypic results (Table 4; Fig. 4). These QTL had LOD values that ranged from 3.38 to 12.84, and they explained 6.24\% to 27.97\% of the phenotypic variation. Furthermore, the FoRsR7.1^59A and FoRsR9.3^59A QTL 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, FoRsR7.1^59A, 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). FoRsR9.3^59A located on R09 also detected in all 3 time trials with phenotypic variation that ranged from 6.24\% to 8.82\%.

To the continuous variation of phenotype in population also imply that the resistant trait is quantitative trait instead of qualitative trait in *R. sativus*.

**Table 2** Details of sequencing reads mapped to the reference genome of radish inbred lines

| Plant samples | YR4     | YR18   |
|---------------|---------|--------|
| Total No. of raw reads | 190,042,688 | 208,986,506 |
| Total raw bases length (bp) | 19,194,311,488 | 21,107,637,106 |
| Total No. of non-redundant reads | 184,883,988 | 204,279,510 |
| Total No. of clean reads | 159,057,674 | 174,640,942 |
| Total bases length of clean reads (bp) | 13,486,715,865 | 14,859,412,353 |
| Total No. of mapped reads | 122,055,397 | 135,002,931 |
| Map reads rate (%) | 76.8 | 77.3 |
| Mapped region (bp) | 284,960,554 | 285,367,161 |
| Genome coverage | ~25.45 × | ~28.04 × |

**Fig. 3** Chromosomal distribution of the SNPs and InDels between ‘YR4’ and ‘YR18’ plant genomes
Candidate gene identification and comparison with previously identified resistant QTL

The FoRsR7.1^{59a} QTL, which was detected repeatedly 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 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 detected in the FoRsR7.1^{59a} 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 S5). 

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^{59a} 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 S5). Both Rs382940 and Rs382200 were expressed only in ‘YR4’ from 0 to 6 days after the inoculation (Fig. 5a).

Rs382950 was highly expressed in ‘YR18’ than ‘YR4’ before inoculation. In ‘YR4’, expression intensity has kept strong over time after inoculation. But, in ‘YR18’, expression level was decreased gradually up to 3rd 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

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**Table 3** Summary of genetic linkage map in *R. sativus*

| Chromosome | Length (cM) | ACMP | BRPGM | EST-SSR | SNP | Total Interval of markers (cM) |
|------------|-------------|------|-------|---------|-----|--------------------------------|
| R01        | 80.11       | 5    | 7     | 4       | 31  | 47                             |
| R02        | 118.39      | 2    | 5     | 4       | 33  | 44                             |
| R03        | 72.82       | 3    | 2     | 5       | 9   | 18                             |
| R04        | 135.86      | 6    | 7     | 2       | 21  | 36                             |
| R05        | 182.75      | 4    | 1     | 9       | 36  | 50                             |
| R06        | 143.41      | 4    | 6     | 0       | 31  | 41                             |
| R07        | 104.70      | 6    | 3     | 2       | 78  | 89                             |
| R08        | 107.51      | 2    | 3     | 8       | 8   | 21                             |
| R09        | 128.68      | 2    | 3     | 1       | 51  | 57                             |
| Total      | 1074.23     | 34   | 37    | 35      | 298 | 403                            |

*a*ACMP cited from Ramchiary et al. (2011)  
bBRPGM cited from Li et al. (2010) and Ramchiary et al. (2011)  
cEST-SSR cited from Shirasawa et al. (2011)  
dSNP designed base on the bi-parental genomes in this study
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 separately, and investigated from the initial time after inoculation (Fig. 5b). In the $Rs_{382950}$, the expression aspects that suddenly disappear from YR18 was reaffirmed in Day 6 condition. Two genes ($Rs_{382850}$ and $Rs_{382860}$) 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 $FoRsR7.159A$ loci. From two major locus, $FoRsR7.159A$ and $FoRsR9.359A$, six FW resistance-specific markers were developed such as RsTN1 ($Rs_{382940}$), RsRLK1($Rs_{382200}$), RsRLK2 ($Rs_{382860}$), RsRLK3 ($Rs_{380550}$), RsTNL1 ($Rs_{495390}$), and RsTNL2 ($Rs_{382950}$) based on sequence variations between the parental lines. Details regarding the primers are provided in Additional file 1 Table S6.

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 F2 population were divided by haplotype, and then investigated the disease resistance with DI in each group. The individuals carrying the YR4-type allele (haplotype ‘a’) had significantly low DI than those with the YR18-type allele (haplotype ‘b’) (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 are useful for enhancing yield and quality, which will increase the economic value of crops (van Buuren 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 QTL 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; Jagannathan 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

### Table 4

| QTL | Linkage group | Confidence interval (cM) | Marker interval | Marker | LOD $R^2$ (%) | Add Dom LOD $R^2$ (%) | Add Dom | LOD $R^2$ (%) | Add Dom | LOD $R^2$ (%) | Add Dom | LOD $R^2$ (%) | Add Dom |
|-----|---------------|--------------------------|-----------------|--------|---------------|------------------------|--------|---------------|--------|---------------|--------|---------------|--------|
| $FoRsR7.159A$ | R7 | 62.70–66.21 | R7 R1176 | 5.17 | 11.69 | 5.16 | 10.17 | 5.16 | 10.17 | 5.16 | 10.17 | 5.16 | 10.17 |
| $FoRsR9.159A$ | R9 | 30.63–33.56 | R9 | 6.10 | 10.81 | 6.10 | 10.81 | 6.10 | 10.81 | 6.10 | 10.81 | 6.10 | 10.81 |
| $FoRsR9.259A$ | R9 | 41.31–46.46 | R9 | 4.52 | 8.18 | 4.52 | 8.18 | 4.52 | 8.18 | 4.52 | 8.18 | 4.52 | 8.18 |
| $FoRsR9.359A$ | R9 | 114.64–128.68 | R9 | 3.38 | 1.41 | 3.38 | 1.41 | 3.38 | 1.41 | 3.38 | 1.41 | 3.38 | 1.41 |
The markers are shown to the right side of linkage group, and genetic distances are represented to the right side of linkage group as centi-morgans (cM). QTL 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.
Fig. 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 59A 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 h, and 1, 2, 3, 6, 12 days.

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 analyzed with one-way ANOVA.
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, F1 plants were highly resistant to FW pathotype 59A, indicating that the resistance is likely controlled as dominant genes in this materials. The DI in the F2 and F2,3 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 was 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 represents 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 and Robin Buell 2012; Fuentes-Pardo and Ruzzante 2017; Jagannathan 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. 2015). The latest genome sequence released by Jeong et al. (2015) 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 predicted genes. In the present study, we re-sequenced the parental sequences enabled us to predict 218,237 SNPs and InDels that were further used to construct linkage groups. Both Rs382850 and Rs382860 are homologous to AT1G73080, which encodes a leucine-rich repeat receptor kinase (LRR-RLK) 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 FoRsR7.159A 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. 2007b, a; Yamada et al. 2016; Jing et al. 2020). The Rs382950 gene belongs to the TIR-NBS-RLR 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 TIR-NBS protein was reported to be induced as a basal defense responses, which can function together in the recognition of pathogens (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 F2 population, we identified and mapped four QTL associated with FW resistance on two chromosomes. Of these QTL, FoRsR7.159A and FoRsR9.359A were considered to be an important major QTL 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.

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Author contribution statement SRC and YPL and designed the study. YM carried out experiments, generated data. SRC, YM analyzed all data, and drafted manuscript. SSC participated in candidate gene identification, writing and editing of the manuscript. LL did marker survey and genotyping. LL, SK, GJC, SML, THG 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.

Declarations

Conflict of interest The authors declare that they have 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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