Government of Saskatchewan
Ministry of Agriculture

ADF Project Final Report Format

1. Project title and ADF file number.

Defining populations of *Plasmodiophora brassicae* with near isogenic *Brassica napus* lines
ADF file number : # 20170055

2. Name of the Principal Investigator and contact information.

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4. Abstract/ Summary: *An outline on overall project objectives, methods, key findings and conclusions for use in publications and in the Ministry database (**Maximum of 500 words** or one* page).

Abstract Content: The abstract addresses the following (usually 1–2 sentences per topic):
- Key aspects of the literature review
- Problem under investigation or research question(s)
- Clearly stated hypothesis or hypotheses
- Methods used (including brief descriptions of the study design, sample, and sample size)
- Study results
- Conclusions

Clubroot disease, caused by *Plasmodiophora brassicae*, pose a serious threat to canola production worldwide including western Canada. Several researchers classified *P. brassicae* into various pathotypes using differential reactions on *Brassica* cultivars such as the Williams Differential, European Clubroot Differential and Canadian Clubroot Differential. Most of the differentials are essentially non-canola crops with unknown genetic information. In this project, we aimed to develop a set of near isogenic spring type *B. napus* lines and define populations of *P. brassicae* collected in western Canada. However, there were very limited genetic studies on resistance to Canadian strains when we initiated the studies in 2014. We therefore proposed to develop *B. napus* isogenic lines with eight CR genes (*CRa, CRb, CRc, CRk, Crr1-Crr4*) from brassica vegetables previously identified by Japanese and Korean scientists. We found that *CRa* and *CRb*, which are identical genes, were effectively resistant to Canadian strains. However, the other six genes were not identified in the mapping populations with Canadian strains. We confirmed *CRa* is the same gene as *Rcr1*/*Rcr2*. Genetic mapping has been performed in several DH populations with introgressed CR genes from turnips, resulting in the identification of four novel genes at *Rcr9* locus and one gene at *Rcr10* locus. In addition, a gene at *RcrM* locus was identified from two mapping populations, one from European canola cv. 'Mendel' and other from intorgressed *B. napus* lines originating from turnip cv. 'Siloga'. Developing the isogenic lines was performed through conventional breeding methods (interspecific crosses, backcrosses and self-pollination), plant tissue culture and marker assisted selection. Approximately 1300 doubled haploid (DH) lines derived from BC2 to BC4 donor plants with clubroot resistance from one *B. napus* and seven *B. rapa* cultivars have been obtained. Genotyping by sequencing was performed in about 500 DH lines. Selection was carried out by testing clubroot resistance to strains collected in western Canada and evaluating morphological traits under greenhouse and field conditions. Finally, a set of isogenic lines carrying eight single race specific genes and three lines carrying more than one genes have been developed. Testing the lines with 36 strains of *P. brassicae* collected on the Prairie was completed. The strains were classified into 28 races, indicating there are very diverse races on canola fields in western Canada. We also found that the lines with the more than one genes exhibited strong resistance to 34 or 35 out of the 36 strains of *P. brassicae*. These lines with broad spectrum of resistance to

clubroot are very valuable to seed companies for developing resistant cultivars. This is the first set of CR near isogenic lines in canola. The lines could replace the current Canadian Clubroot Differential set and potentially revolutionize the clubroot pathotying system in the world.

5. **Extension Messages:** *key outcomes and their importance for producers/processors and the relevant industry sector (**3-5 bullet points** **in lay language).***
   - More than 1200 canola DH lines with introgressed CR from brassica vegetables have been developed, which are very valuable resources for identifying novel genes and selecting novel breeding lines for resistance to clubroot in the future studies.
   - Next generation sequencing technologies were used for genetic mapping of CR genes, which has greatly accelerated CR gene identification.
   - Canola lines with broad spectrum of clubroot resistance derived from three sources of turnips have been developed. These lines are very valuable for developing resistant cultivars by canola breeders.
   - The first set of near isogenic lines containing eight single CR genes in brassica crops have been developed. The lines could replace the current Canadian Clubroot Differential set and also monitor race change in the pathogen race structure.
   - Thirty-six strains of *P. brassicae* collected in western Canada were classified into 28 races. This may only be the tip of the iceberg for the pathogen population as more races will be identified if more strains are tested.

6. **Introduction:** *Brief project background and rationale (**Maximum of 1500 words or 1.5-3 pages**).*

Clubroot disease, caused by *Plasmodiophora brassicae*, poses a serious threat to canola production in western Canada. Several research groups have classified *P. brassicae* into various pathotypes based on differential reactions on brassica cultivars. There were three main differential systems in use: the differential set of Williams (1966), which consists of two cabbage and two rutabaga cultivars, the European Clubroot Differential (ECD) set (Buczacki et al., 1975), which consists of five cultivars of each of *B. rapa*, *B. napus* and *B. oleracea*, and the differentials used by Somé et al (1996) with seven lines of *B. napus* and two lines of *B. oleracea*. Each differential set has its advantages and disadvantages (Strelkov et al. 2012). The differential set of Williams has been usually used in Canada because of its simplicity. However, the pathotype designations likely do not reflect the full pathogenic diversity of *P. brassicae* on canola (Howard et al. 2010). Therefore, development of a new differential system is required. A Canadian Clubrot Differential (CCD) set, which consists of 13 brassica lines including William's, Somé's and some of the ECD lines, was proposed and has been used for pathotype classifications in Canada recent years (Strelkov et al. 2016). Strains of *P. brassicae* collected in the canola fields on the prairie have been classified into more than 30 pathotypes using the CCD. However, most of the CCD lines are non-canola crops, belong to vegetable or fodder brassicas. In addition, the pathotypes defined by the differential sets do not reflect the variation of race-specific genes in *P. brassicae* populations on canola. It is very difficult to use the classification of pathotypes to guide the deployment of CR genes and gene rotation, a strategy for effective control of the disease. A set of spring *B. napus* near-isogenic lines (NILs) containing single CR genes is ideal for differentiating races of *P. brassica.*

Genetic mapping of clubroot resistance (CR) genes have been extensively carried out in *B. rapa* and *B. oleracea*. Genetic analysis of the CR genes in *B. oleracea* indicates that they are quantitative traits, which is usually controlled by quantitative trait loci (QTLs) with minor effects. It is difficult to use breeding lines or cultivars with minor QTLs for classification of the clubroot pathogen into races although > 20 QTLs were mapped in different linkage groups of *B. oleracea*. When Dr. Fengqun Yu proposed the development of NILs as a new Canadian clubroot differentiate set in 2013, eight CR genes (*CRa*, *CRb*, *CRc, CRk*, *Crr1*, *Crr2*, *Crr3* and *Crr4*) from European fodder turnips had been identified and mapped by scientists in Japan and Korea. Afterwards, the Clubroot Genetics Group led by Dr. Yu at the Saskatoon Research and Development Centre, Agriculture and Agri-Food Canada (SRDC, AAFC) identified *Rcr1* in pak choy cultivar 'Flower Nabana' (Chu et al., 2014; Yu et al., 2016) and *Rcr2* in Chinese cabbage cultivar 'Jazz' (Huang et al., 2017). In addition, two novel genes (*Rcr8* and *Rcr9*) resistant to pathotype 5X of *P. brassicae* were genetically mapped into chromosomes A02 and A08 respectively from a *B. rapa* breeding line

T19 (Yu et al., 2017). Identifying clubroot resistance genes in brassica species have been also carried out in Dr. Habibur Rahman's group at the University of Alberta (Fredua-Agyeman et al., 2016; Hasan et al., 2016) , Dr. Genyi Li's group at University of Manitoba (Gao et al., 2014) and Dr. Sheau-Fang Hwang's group (Zhang et al., 2016). It is very likely that the resistance gene identified by Fredua-Agyeman et al. (2016) and Zhang et al. (2016) is the same gene that has been used in the first generation of Canadian canola cultivars originating from European canola cultivar 'Mendel', herein designated as *RcrM*.

A four-year project (#20130122) on "Developing near-isogenic *Brassica napus* lines for differentiating pathotypes of *Plasmodiophora brassicaehas*" had been funded by ADF, SaskCanola and WGRF until March 31, 2018. By the end of the project, most of the introgression lines were in the generation BC3, and the development of doubled haploids (DH) for selected introgression lines was just initiated. The objectives for the current project were to: 1) complete the development of the lines proposed in the project #20130122; 2) develop breeding lines carry *RcrM*; 3) develop breeding lines carrying *Rcr8* and *Rcr9*; and 4) define the populations of *P. brassicae* collected in western with the newly developed lines.

7. Objectives and the progress towards meeting each objective.

Objectives **(Please list the original objectives and/or revised objectives if Ministry-approved revisions have been made to original objective. A justification is needed for any deviation from original objectives)**	**Progress** **(e.g. completed/not completed)**
a) To complete development of a set of single clubroot-resistance-gene *Brassica napus* lines.	Completed. More than 1200 DH lines were developed from BC2-BC4 plants originating from *B. rapa* cultivars or breeding lines (Siloga, ECD01/Debra, Milan, ECD02, FN, 69-6992) potentially carrying *CRa*, *CRb*, *CRc, CRk*, *Crr1*, *Crr2*, *Crr3* and *Crr4*. The relationship for the genes closely linked or co-located into chromosome A03 was determined though molecular cloning. Genotyping by sequecing (GBS) and genetic mapping was performed in most of the DH popultions. Novel genes ($Rcr9^{ECD01}$, $Rcr10^{ECD01}$, $Rcr9^{6992}$, $Rcr9^{Siloga}$ was identified. Selected lines were evaluated in greenhouse and field conditions. Five NILs *Rcr1*, $Rcr9^{ECD01}$, $Rcr10^{ECD01}$, $Rcr9^{6992}$, $Rcr9^{Siloga}$ were chosen.
b) To transfer a resistance gene (*RcrM*) originating from European canola cultivar 'Mendel' into a spring-type *B. napus* line DH16516.	Completed. A CR gene *(RcrM*) different from *Rcr1* was confirmed through molecular cloning. The gene at *Rcr1* locus showed 88% similarity with *Rcr1*. *RcrM* was identified in the DH population consisting of 20 lines from the 'Mendel' cross and the DH population consisting of 199 DH lines from the 'Siloga' cross. One of the best lines with *RcrM* was added into the NIL set.
c) To transfer *Rcr8* and *Rcr9* from *B. rapa* into *B. napus* line DH16516.	Completed. A NIL line carrying *Rcr8* was chosen in the DH population consisting of 15 DH lines from the T19 cross. Genetic mapping indicated that *Rcr9* was likely the gene same as $Rcr9^{6992}$ in turnip line 96-6992.
d) To define the populations of *P. brassicae* with the newly developed near isogenic lines.	Completed. A total of 76 strains in canola field in western Canada were obtained with 36 successfully propagated on canola line DH16516. Twelve lines including the susceptible recipient line DH16516 were tested with the 36 strains. The clubroot stains were classified into 28 races.

Please add additional lines as required.

8. **Methodology:** *Specify project activities undertaken during the entire project period (without referring to previous progress report). Include approaches, experimental design, methodology, materials, sites, etc.* ***(Maximum of 5 pages)***

Plant materials

Donor Line	CR gene	Chromosome	Generation
Turnip Milan White	*Crr3*	A03	BC3
ECD01	*CRb*	A03	BC3
ECD02 (Gelria)	*CRa; CRb*	A03	BC3
Debra	*CRc; CRk*	A02, A03, A08	BC2/BC3
Flower Nabana	*Rcr1*	A03	BC4
Jazz	*Rcr2*	A03	BC4
Siloga	*Crr1, Crr2, Crr4 (RcrM, Rcr9)*	A08, A01, A06, A03	BC3
Mendel	*RcrM*	A03	BC2
T19/6992	*Rcr8* and *Rcr9*	A02, A08	BC1

Method for pre-breeding

Conventional breeding methods such as crossing and backcrossing, and molecular marker-assisted selection (MAS) were used in this project. Introgression of the resistance genes from the *B. rapa* into *B. napus* followed the strategies described previously by Yu et al (2012). Resistant plants of the *B. rapa* donors were crossed with *B. napus* DH16516. The resulting F1 plants (allotriploid) were crossed into the susceptible background to back cross generations 2 to 4 (BC2 to BC4).

Intogression of *RcrM*, which was present in the first generation of Canadian canola resistant cultivars, was performed by crossing DH16516 with European canola cultivar 'Mendel' and backcrossing with DH16516 into BC2. One BC2 plant carrying *RcrM* on chromosome A03 through MAS and testing for resistance to pathotype 3H was used for the donor or microspore culture.

Microspore culture was performed to produce DH plants following the protocol as described by Coventry et al (1998) with some modifications. DH plants grew in a greenhouse at SRDC, AAFC.

Cloning of the candidate CR genes *Rcr2, RcrM* at *Rcr1/CRa* locus

Based on our prior work, we have mapped a single dominant clubroot resistant (CR) gene on chromosome A03. For cloning of the candidate CR gene, we first designed a series of specific primers using the published *B. rapa* reference genome from an online database (BRAD), and then performed the classical molecular cloning on a collection of resistant cultivars of *B. rapa*, JNC (*Rcr2*), and Mendel (*RcrM*) at *Rcr1/CRa* locus. The susceptible *B. rapa* cultivar of ACDC was also included as a control. After PCR amplification, PCR products of expected size (about 4600 bp) were cloned using TOPO Cloning Kit (Invitrogen), and selected clones from each cultivar were sent for full sequencing.

DNA sequencing and alignment of reads to a reference genome

GBS was performed in 400 DNA samples for the project. DNA was extracted from young leaves of each of DH lines and parental lines following DNeasy Plant Mini Handbook from QIAGEN. GBS of the DNA samples and two replications of the parental cultivar were usually performed on an Illumina platform with pair-end sequencing at BGI Americas Corp (Cambridge, MA, USA). DH16516 is an important *B. napus* canola recipient line for introgression of clubroot resistance at AAFC, Saskatoon, so whole-genome sequencing of the line had already been performed at

Plant Biotechnology Centre (Saskatoon, SK, Canada) as part of the generation of a new reference genome (unpublished data). The short reads from the whole-genome sequencing data was used for the study. The program SeqMan NGen 15 (DNASTAR, Madison, WI, USA) was used for short read assembly. 'Whole genome DNA-Seq/Genotyping' assembly workflow, 'Reference based assembly-normal workflows' assembly type, 'Automatic Mer size, Automatic Minimum match percentage, High Layout stringency and Medium SNP filtering stringency' assembly options were chosen. Short reads from each of the samples, parental DH16516 and the combined two replicates of ECD01 were aligned to *B. napus* reference genome for cv 'Darmor-*bzh*' v4 downloaded from https://www.genoscope.cns.fr/brassicanapus/data/ and the newest version(v3.0) of *B. rapa* genome was downloaded at http://brassicadb.org/brad.

Identification of variants, variant filtering, construction of linkage map and QTL mapping

Identification of variants (SNPs and InDels) in the DNA sequences of each sample relative to the reference genome of *B. napus* 'Darmor-*bzh*' was performed using SeqMan Pro 15 (DNASTAR, Madison, WI), but only SNPs were used for further study. Comparison of the variants among the DH samples was carried out using Qseq 15 (DNASTAR).

GBS-SNP sites were named based on the reference genome (DM: 'Darmor-*bzh*'), the A-genome chromosome (A01 to A10) and position on the reference chromosome sequence. A SNP site was called in a given sample at following criteria: depth > 5, Q > 30 and SNP percentage >50%. Since the recipient parent DH16516 and the DH lines were DH lines, all SNP sites should theoretically be homozygous. After filtering, heterozygous genotypes in the parental line DH16516 and the DH lines, and monomorphic phenotypes between the parents or among the individuals were removed.

The remaining SNP sites after filtering were further analyzed using JoinMap 4.1 (Van Ooijen, 2011). SNP alleles from the resistant parent were scored as 'B' and those from the susceptible parent (DH16516) as 'A'. Marker orders and positions in the genetic map were determined using maximum likelihood in Kosambi's model with a minimum logarithm of the odds (LOD) values of 10. Only SNP sites that could be assigned into the 10 chromosomes of the A-genome at LOD scores of 10.0 were kept. The set of filtered SNP sites obtained was used for binning of redundant markers, construction of linkage map, and mapping of QTLs for resistance to clubroot using the QTL IciMapping Inclusive Composite Interval Mapping (ICIM) method (Meng et al., 2015). A linkage map was drawn using Mapchart 2.1 (Voorrips, 2002) based on the genetic location determined with QTL IciMapping. The LOD score threshold was set using a 1,000-permutation test with a Type I error of 0.05 for QTL declaration. The QTL effects were estimated as phenotypic variation explained (PVE) and additive (Add) values by each QTL.

Identification of genes in the target regions of the *B. napus* 'Darmor-*bzh*' reference genome

Gene annotation was analyzed with Blast2GO (Conesa et al., 2005) using coding sequences (CDS) of the genes in each of the QTL target regions from 1 Mb upstream to 1 Mb downstream of the SNP markers in the peak regions as determined by ICImapping. Genes related to disease resistance and defense responses were identified using Blast2GO information of the gene description and gene ontology. The most probable Arabidopsis homolog corresponding to each disease resistance gene and the class of disease resistance proteins were obtained using the CDS of the disease resistance gene in the *B. napus* by Blast search at **www.arabidopsis.org**.

Mapping of QTLs with bulked segregant analysis (BSA)

BSA has been used to detect molecular markers linked to traits of interest, such as disease resistance (Michelmore et al., 1991). In BSA, bulks of plants with contrasting phenotype are generated. Our previous studies showed that a gene could be genetically mapped by identifying the though the identification of the percentage of polymorphic variants in a genome using BSA (Yu et al . 2016; Dakouri et al. 2018; Huang et al. 2019; Karim et al. 2020).

DH lines were selected to form a R bulk and a S bulk based on their phenotypes using SNP marker-assisted selection. GBS data from the R and S bulks was aligned onto the *B. napus* reference genome separately using SeqMan NGen 15 (DNASTAR). Mapping of the QTLs was performed using the PPV method described by Yu et al. (2016) and Dakouri et al. (2018).

Search for the syntenic regions of identified QTLs in *B. rapa* 'Chiifu' reference genome

The *B. rapa* reference genome v3.0 (Zhang et al., 2018) was downloaded from http://brassicadb.org/brad/downloadOverview.php. DNA sequences of the QTL target regions from the A-genome of *B. napus* were aligned into the *B. rapa* genome using Megalign Pro 15 with MAUVE (DNASTAR).

Isolates of *P. brassicae* and evaluation of plants for resistance to clubroot

Clubroot strains collected in canola fields in Alberta, Saskatchewan and Manitoba were provided by Drs. Stephen Strelkov, Alireza Akhavan / Barbara Ziesman and Lee Anne Murphy respectively. A total of 76 strains were obtained with 36 successfully propagated in canola line DH16516. Fresh and clean clubbed roots harvested at 4–5 weeks after inoculation of each strain were cut into smaller pieces with scissors, macerated in distilled water for 1–2 hours and blended in a blender at high speed for 2 min. After filtering through eight layers of sterile cheesecloth, resting spores extracted from the clubbed roots were adjusted to a concentration of 1.0 x 10^7 resting spores/ml in distilled water for plant inoculation.

A highly efficient method for testing plants for resistance to clubroot was developed in Dr. Yu's group. Seeds each line were sown into Sunshine #3 soilless mix (Sun Gro Horticulture Canada Ltd.; Seba Beach, AB) with Osmocote (Everris NA Inc.; Dublin, OH, USA) in 32-pot inserts held by trays (The HC Companies; Twinsburg, OH, USA). About 4 L water was added to each tray to soak the soilless mix overnight. Seven days after planting, inoculation was performed by adding 15 ml of inoculum (1 x 10^7 spores/ml) into each pot with 6–9 seedlings of each line. The inoculated plants were grown in a growth chamber set at 22/18°C day/night temperature with a 16-h photoperiod. The canola cultivar '45H29' (Pioneer) or a DH line, NRC11-24 (Nutrien Ag Solutions, Saskatoon, SK) that carry *RcrM* and the universal susceptible line DH16516 were included as controls. Six weeks after inoculation, plants were pulled and the roots were examined for clubroot symptoms.

Clubroot severity was evaluated on a 0 to 3 scale, where 0 = no clubbing, 1 = a few small clubs, 2 = moderate clubbing, and 3 = severe clubbing. A disease severity index (DSI) was calculated for each line using the method of Horiuchi and Hori (1980) (Horiuchi and Hori, 1980):

$$\text{DSI} = \frac{\sum (\text{rating class}) \times (\#\text{ plants in rating class})}{\text{total }\#\text{ plants in treatment} \times 3} \times 100$$

Each line with a resistance response (DSI ≤ 30%) in the initial study was re-assessed two more times. Each of these repetitions provided a similar result in most cases. For those lines with inconsistent results, the highest DSI among the three repetitions of the assessment was considered to be the most accurate and was used to characterize the resistance response of the line. DH lines with DSI ≤ 30% were classified as R and those lines with DSI > 30% as S lines.

Kompetitive Allele Specific PCR (KASP)

Selected SNPs identified in the target region for each gene or QTL were confirmed using the KASP method (http://www.lgcgroup.com/) following the manufacturer's instructions. Polymerase chain reactions were performed in a StepOne Plus Real Time PCR System (Applied Biosystem, Mississauga, ON). Linkage analysis with the confirmed SNP markers was performed using KASP analysis and the phenotypes determined based on the mean DSIs of the four pathotypes using JoinMap 4.1 (Van Ooijen, 2001).

Field experiments

One field trial consisting of 340 lines with single rows of each test entry was conducted at AAFC Saskatoon Research Farm in 2020. The plot included check (the recurrent parent DH16516). Days to flowering and morphological similarity to their recurrent parent were observed. Selected lines with similar days to flowering to their recurrent parent were tented for seed production.

9. Results and discussion: *Describe and discuss the results accomplished during the entire project period under each objective listed under section 7. The results need to be accompanied with pertinent tables, figures and/or other illustrations. Provide discussion necessary to the full understanding of the results. Where applicable,*

*results should be discussed in the context of existing knowledge and relevant literature. Detail any major concerns or project setbacks. (**Maximum of 30 pages of text not including figures or tables**)*

1) Development of homozygous lines through conventional breeding and microspore culture

The first objective for this project was to develop a set of isogenic lines containing *Rcr1* and *Rcr2* (identified by our group), *Crr1-Crr4*, *CRa*, *CRb*, *CRc* and *CRk* (identified by Japanese and Korean scientists). Homozygous lines in BC4S2 carrying *Rcr1* and *Rcr2* respectively had been obtained through conventional interspecific crosses, backcrossing until BC4 and self-pollination to produce BC4S2 in the project ADF#20130122 . In this project, we focused on developing DH lines from selected plants in BC2 and BC3 (Table 1). Embryos produced from single microspore were transferred into modified B5 medium to generate plantlets (Figure 1A). Plantlets then were transplanted into soilless mix and kept in a growth chamber (Figure 1B) until 1-3 new true leaves showed up in each plant (about 2-3 weeks). The survived plants were transferred into greenhouse for seed production (Figure 1 C & D). A total of **1259** DH lines have been produced from 8 donor lines (Mendel, Siloga, ECD01/Debra, Milan, ECD02, FN, 69-6992, T19) (Table 1).

2) Morphological selection in greenhouse and field conditions

Morphological traits such as date to flowering, requirement of vernalization, plant vigour were observed for selected DH lines. Here is an example of 59 DH lines in the population Y154 (Table 1) for examined in greenhouse at the SRDC, AAFC in 2018 (Figure 2). The DH lines with similar days to flowering to the recurrent parents were further evaluated in the field condition.

A field trial consisted of 323 CR homozygous lines and 17 rows of DH 16516 was performed (Figure 3). Most of the CR lines looked similar to their recurrent parent morphologically. Days to flowering were in a range of 49 to 52 in DH16516. Among the 323 lines, there were 250 lines (77%) with days to flowering in the same range as that of DH 16516. Isolation tents were put for 146 out of the 250 lines and seeds were obtained from all the lines.

3) Determining the relationship of *Rcr1 /CRa*, *Rcr2* and *RcrM*

Two CR genes *Rcr1* and *Rcr2* for resistance to Canadian clubroot pathotypes were identified from bok choy cv. 'Flower Nabana' (Chu et al., 2014; Yu et al., 2016) and Chinese cabbage cv. 'Jazz' (Huang et al. 2017) respectively. The genes were co-located into chromosome A03 of *B. rapa*. Furthermore, *CRa* for resistance to Japanese clubroot strains had been identified previously. Both *Rcr1* and *Rcr2* were mapped into the genetic region close to *CRa* that had been isolated from Chinese cabbage line of *B. rapa*. It encodes Toll-Interleukin-1 receptor / nucleotide-binding site / leucine-rich-repeat (TIR-NBS-LRR; TNL) proteins (Ueno et al. 2012). We performed map-based cloning of *Rcr1* from bok choy cv.'Flower Nabana' under the support of AAFC Genomics Initiative, leading to the identification of several genes encoding TNL proteins in the *Rcr1* target region. Pathogenicity tests revealed that one of the cloned TNL genes at *Rcr1* locus was identical to *CRa* (Table 1). When delivered into the fragment into susceptible canola line DH12075 via *Agrobacterium*-mediated transformation, the gene has rendered this canola line strong resistance against pathotype 3H of *P. brassicae*. Therefore, *Rcr1* and *CRa* are the same gene. To address the relationship of *Rcr2* with *Rcr1/CRa*, we performed isolation of *Rcr2* from Chinese cabbage cv. 'Jazz', resulting in the identification of an identical DNA fragment to *Rcr1/CRa* for *Rcr2* (Table 1). Furthermore, we confirmed the presence of *CRa* in turnip cv. ECD02.

RcrM originating from 'Mendel' was mapped into the same region as *CRa* in chromosome A03 (Fredua-Agyeman et al. 2016, Zhang et al. 2016). Molecular cloning indicated that similarity of the fragment in the 'Mendel' with *CRa* was 88.3% and it was not identical to *Rcr1/Rcr2/CRa*. This result indicates *RcrM* is different from *CRa* (Table 1). However, the resistance phenotype with the fragment needs further characterization with transformation experiment.

4) Identification of CR genes in turnip cv. 'Debra' (ECD01) and development of isogenic lines carrying *Rcr9*^{ECD01} and *Rcr10*^{ECD01}– a case study with identification of CR genes in the DH lines through genotyping by sequencing (GBS)

'Debra' was included in the ECD set as a differential line ECD01 (Buczacki et al., 1975; Diederichsen et al., 2009). Therefore, 'Debra' and 'ECD01' are the same cultivar. *CRb*, a CR gene identified in a Chinese cabbage cv.

'CR Shinki' and two CR genes in Chinese cabbage cv. 'CR Kanko', *CRk* and *CRc,* were derived from ECD01 (Piao et al., 2004) and 'Debra' (Sakamoto et al., 2008) respectively. Our original target was to develop *B. napus* isogenic lines carrying *CRb*, *CRc* and *CRk* introgressed from 'ECD01'. As there were no strains for identifying the genes available for the project and no molecular makers linked to each of the genes could be used for the project, we developed DH populations derived from interspecific crosses and performed genetic mapping of the genes from the populations. Five DH populations in BC2 or BC3 were developed (Table 5). Genetic mapping was performed in one (Y154) of the populations consisting of 84 DH lines. Two major QTLs in *B. napus* lines with introgressed clubroot resistance from turnip cv. ECD01 from the BC2 DH population have been identified. This research has been published into **Front. Plant Sci.** https://doi.org/10.3389/fpls.2021.785989. Please see the results below.

4.1 Resistance to clubroot in the parental lines and the BC2 DH population consisting of 84 lines (population Y154)

The clubroot reaction of the parental lines (ECD01 and DH16516), controls and the DH population were assessed against pathotypes 3A, 3D, 3H and 5X (Table 3). As expected, ECD01 was highly resistant to all pathotypes (0% DSI), DH16516 was highly susceptible (100% DSI) and '45H29' was resistant to pathotype 3H only (Figure 4, Table 3). The F_1 plants from the interspecific crosses of DH16516 x ECD01 with highly resistant to pathotype 3H (0% DSI), which was the predominate pathotype in Canada before the emergence of the 3A, 3D and 5X. Clubroot severity in response to inoculation with each pathotype in the DH population could be divided into two classes: resistant (R) lines with DSI ≤ 30% and susceptible (S) lines with DSI > 30% (Figure 5). The segregation ratio of R and S was calculated and goodness of fit was tested with a $\chi 2$ test using Microsoft Excel software. Of the four pathotypes, segregation of R and S best fit a 1:3 ratio for pathotype 3A and a 3:1 ratio for pathotypes 3D, 3H and 5X. These results indicated that resistance to pathotype 3A was controlled by two genes in complementary action, and resistance to pathotypes 3D, 3H and 5X by two genes in duplicate action.

Correlation coefficients among the disease severity index values for the pathotypes ranged from 0.55 to 0.81, but all were significant at $P < 0.01$ (Table 4). This indicated that the genes for resistance to the different pathotypes were likely controlled by the same genes or tightly linked genes.

4.2 Alignment of DNA short reads into the *B. napus* genome

Since clubroot resistance in the DH population originated from the A-genome of *B. rapa* cv ECD01, only A-genome DNA sequences in the reference genome *B. napus* 'Darmor' v4.1 were used for alignment of DNA short reads and discovery of DNA variants (SNPs and InDels). About 219.9 million (M) short reads were obtained from whole-genome sequencing from DH16516 and 53.1% of the reads were assembled into the reference A-genome; 13.8 M sequences were obtained from GBS of ECD01 and 70.9% were assembled into the reference A-genome. A total of 355.3 M short reads from 84 DH lines were obtained, ranging from 0.81 to 11.67 M sequences per line. The mean number of reads aligned into the reference genome from each line was 2.3 M (range 0.46 to 5.22 M) and 54.7% were assembled into the reference A-genome.

4.3 Identification of polymorphic SNP sites and QTL analysis

After the initial filtering, 429 polymorphic SNP sites remained, distributed to 9 of 10 chromosomes of the reference genome of 'Darmor'. No polymorphic markers were identified from chromosome A06. There was no correlation between chromosome size and the number of SNP markers identified (r = -0.092) in the population. To remove redundant markers, the 429 SNP sites were further filtered using the BIN function in ICIMapping, which left only 260 non-redundant SNP sites. A genetic map of the nine chromosomes of the A-genome was constructed from the distributed SNP sites. The length of each chromosome ranged from 0 (chromosome A06) to 471.8 cM (A01), with an average length of 85.3 cM. Chromosome A01 was much longer than the other linkage groups. The number of SNP sites per chromosome ranged from 0 (A06) to 152 (A01), with a mean of 26 SNPs per chromosome. The SNP interval of each chromosome ranged from 0.8 to 4.8 cM, with a mean of 3.3 cM.

Mapping of the QTLs was performed using the linkage map and trait values for resistance to each pathotype (3A,

3D, 3H and 5X). Two QTLs were identified: a QTL designated as *Rcr10*^{ECD01} on A03, with a peak at the SNP markers DM_A03_12570715 and DM_A03_10873502; a QTL designated as *Rcr9*^{ECD01} (Figure 6), located near the previously identified genes *Rcr9* and *Rcr9*^{wa} (Yu et al., 2017;Karim et al., 2020) on A08, with a peak at DM_A08_10325589 and DM_A08_10529713 (Table 3). Resistance to pathotypes 3A, 3D and 3H was associated with the two QTLs (*Rcr10* ^{ECD01} and *Rcr9*^{ECD01}), but resistance to 5X was only associated with *Rcr9*^{ECD01} (Table 5). LOD, PVE, Add values and confidence interval (CI) from the estimated QTL position varied between the QTLs, ranging from 5.2 to 12.2 for LOD, 16.2 to 43.3% for PVE, 14.6 to 25.4 for Add and 1.5 to 12 cM for CI (Table 5). The values of Add for the two QTLs were positive, indicating that the resistant loci were derived from the resistant parent ECD01.

4.4 Identification of disease resistance genes and genes related to plant defense response

Searches for candidate genes for *Rcr10* ^{ECD01} and *Rcr9* ^{ECD01} that encoded disease resistance proteins and defense-related genes were performed using CDS of the reference genome in the target region including 1 Mb up- and down-stream of the left and right markers (Table 5).

Rcr10 ^{ECD01}, which was responsible for resistance to pathotype 3A, 3D and 3H, was mapped into chromosome A03, with a peak at SNP markers DM_A03_10873502 and DM_A03_12570715 (Table 5). There are 676 genes in this 3.7 Mb region. Among the genes, one gene (*BnaA03g25330D*) encoded a disease resistance protein (Table 6) and 24 genes encoded proteins with function related to plant defense response. *BnaA03g25330D* is homologous to the Arabidopsis gene *AT5G22690*, which encoded a TNL protein (Table 6).

Rcr9^{ECD01}, which was responsible for resistance to all four pathotypes, was mapped into chromosome A08, with a peak at SNP markers DM_A08_10325589 and DM_A08_10529713. There were 338 genes in this 2.2 Mb region (Table 6). Two genes (*BnaA08g10100D* and *BnaA08g11840D*) encoded disease resistance proteins and *BnaA08g10100D* was homologous to the previously cloned resistance gene *Crr1*. *BnaA08g10100D* and *BnaA08g11840D* were homologous to the Arabidopsis genes *AT5G11250* and *AT4G33300*, respectively. *AT5G11250* encodes an atypical TNL protein and *AT4G33300* encodes a member of the activated disease resistance 1 family nucleotide-binding leucine-rich repeat immune receptors (Table 6). Also, this region contained 10 genes that encoded proteins with defense-related function.

4.5 Confirming the QTL intervals with BSA

Of the 84 DH lines, 19 lines were resistant to almost all the pathotypes. They all carried alleles from the resistant parent ECD01 (SNP genotype 'B') with *Rcr10*^{ECD01} (DM_A03_10873502 and DM_A03_12570715) and *Rcr9*^{ECD01} (DM_A08_10325589 and DM_A08_10529713). Also, 17 lines were susceptible to almost all the pathotypes and all of them carried alleles from the susceptible parent line DH16516 (SNP genotype 'A') for the two QTLs. As a result, the R bulk was formed from the 19 R DH lines while the S bulk was formed from the 17 S DH lines for the BSA.

A total of 93.5 M short reads from the R bulk and 69.4 M short reads from the S bulk were aligned into the *B. napus* reference genome. A PPV peak (25–30%) occurred within the physical interval 9–14 Mb on chromosome A03 and other peak (25–36%) within the physical interval 9–12 Mb on chromosome A08 (Figure 7), which indicated that *Rcr10*^{ECD01} and *Rcr9*^{ECD01} resided in the intervals of chromosomes A03 and A08, respectively. This result is consistent with the that from the above QTL analysis.

4.6 Search for the syntenic regions of the QTLs in the *B. rapa* 'Chiifu' reference genome

Most of the genes or QTLs for clubroot resistance in *Brassica* species containing the A-genome that have been identified were from *B. rapa*. In this study, the DH population was developed with introgression of QTLs from *B. rapa*, so the QTL target regions of chromosome A03 and A08 of *B. napus* were compared with those of *B. rapa*.

The *Brassica rapa* reference genome 'Chiifu' v3.0 is the most recent version available for the 'Chiifu' reference genome (Zhang et al., 2018). The 3.7 Mb region from 9.8 to 13.5 Mb of *B. napus* chromosome A03, which included a fragment of the markers DM_A03_10873502 and DM_A03_12570715 for *Rcr10*^{ECD01}, was homologous to the region 11.0 to 16 Mb of 'Chiifu' A03 (Figure 6). *Rcr9*^{ECD01}, located on the 2.2 Mb length from

9.3 to 11.5 Mb of *B. napus* chromosome A08, which included SNP markers DM_A08_10325589 and DM_A08_10529713, was homologous to the region 12.0 to 14.5 Mb of A08 in *B. rapa* 'Chiifu' (Figure 8).

4.7 Discussion

Two QTLs for resistance to the four pathotypes of *P. brassicae* derived from *B. rapa* ECD01 were transferred to, identified and mapped in a DH population of *B. napus*. The DH population segregated in a 1:3 (R:S) ratio for resistance to pathotype 3A. This indicated that resistance to pathotype 3A was controlled by two genes in complementary action. The segregation ratio for resistance to pathotype 3H was 3:1, which was also the most likely fit for pathotypes 3D and 5X. This indicated that resistance to all three pathotypes in the DH population was controlled by two genes in duplicate action. Two QTLs, $Rcr10^{ECD01}$ and $Rcr9^{ECD01}$, for resistance to pathotypes 3A, 3D and 3H were identified, which was consistent with the genetic analysis of phenotype ratios. However, only one QTL, $Rcr9^{ECD01}$, was identified for resistance to 5X, although the segregation ratio was close to 3:1. This inconsistency merits further investigation.

In general, strong resistance to clubroot pathotypes is controlled by single dominant genes such as *Rcr1–Rcr7*. Two genes in duplicate action (*Rcr8* on chromosome A02 and *Rcr9* on chromosome A08 from *B. rapa* line T19) the confer resistance to pathotype 5X were reported previously (Yu et al., 2017). Similarly, a previous study indicated that neither *Crr1* nor *Crr2* on their own conferred resistance to Japanese strain 'Wakayama-01' of *P. brassica*; resistance was only expressed when resistance alleles were present at both loci (Suwabe et al., 2003). In the current study, the QTL for resistance to pathotype 3A derived from ECD01 may behave in a similar manner to *Crr1* and *Crr2.*

In the current study, the target region for $Rcr10^{ECD01}$ was defined into 9.8–13.5 Mb of *B. napus* chromosome A03 using QTL analysis. Similar interval (9–14 Mb) for $Rcr10^{ECD01}$ was obtained using identification of the PPV with BSA. The region for $Rcr10^{ECD01}$ in *B. napus* was homologous to the 11.0–16.0 Mb region of A03 in the *B. rapa* 'Chiifu' v3.0. This was a distinct genetic region from *Rcr1*, *Rcr2*, *Rcr4* and *Rcr5* for resistance to pathotypes of *P. brassicae* (Figure 4). The genes *Rcr1*, *Rcr2* and *Rcr4,* which confer resistance to pathotypes of *P. brassica,* have previously been mapped into chromosome A03 of *B. rapa* 'Chiifu' v3.0 at ~25 Mb region (Chu et al., 2014;Yu et al., 2016;Huang et al., 2017), while *Rcr5* was also mapped at ~24 Mb region in that chromosome (Huang et al., 2019) (Figure 4). *Rcr1*, *Rcr2* and *Rcr4* were subsequently co-localized with the cloned CR genes *CRa* / CRb^{kato} (Ueno et al., 2012;Hatakeyama et al., 2017) while *Rcr5* was located in a region close to *CRa* /CRb^{kato} . In addition, resistance genes *Rcr1*, *Rcr2*, *Rcr4* and *Rcr*5 were identified for resistance to pathotype 3H, not for 3A, 3D or 5X. Several CR genes or QTLs, such as *PbBa3.2* (Chen et al., 2013), *CRd* (Pang et al., 2018), *Crr3* (Hirai et al., 2004) and *CRk* (Sakamoto et al., 2008) for resistance to clubroot strains collected from Japan and China, have been mapped into the regions different from *CRa*/CRb^{kato}. Similarly, *BraA.CR.c* for resistance was mapped into chromosome A03 in turnip cvs. ECD01, ECD02 and ECD04 (Hirani et al., 2018). The relationship of $Rcr10^{ECD01}$ to these previously identified genes needs to be determined. Also, *CRb* was identified in a Chinese cabbage cv 'CR Shinki' that was originally derived from ECD01 for resistance to *P. brassica* strains collected in Korea (Piao et al., 2004). It was located in a genetic region close to *CRa* /CRb^{kato}. However, no QTL in the *CRb* region was identified in this study.

A QTL, identified and designated as $Rcr9^{ECD01}$ (because it was mapped into the genetic region of *Rcr9* and was originally derived from *B. rapa* cv ECD01), conferred resistance to all four pathotypes (3A, 3D, 3H and 5X) assessed in this study. $Rcr9^{ECD01}$ was located on the 2.2 Mb length from 9.3 to 11.5 Mb of *B. napus* chromosome A08 using QTL analysis. The $Rcr9^{ECD01}$ interval was confirmed through the identification of PPV with BSA, located in the physical interval 9-12 Mb. The region of $Rcr9^{ECD01}$ in *B. napus* corresponded to 12.0 to 14.5 Mb of A08 in *B. rapa* 'Chiifu' v3.0 (Figure 4).

Previously, our laboratory had identified *Rcr9* for resistance to pathotype 5X in *B. rapa* breeding line T19, which originated from German turnip cv. 'Pluto' (Yu et al., 2017). The proposed position of *Rcr9* spanned a large interval (6.48 Mb) of chromosome A08, including the genome region of *Rcr3* and $Rcr9^{ECD01}$. However, several breeding lines that carried *Rcr9* were resistant to 5X, but not to 3A, 3D (Yu, unpublished) and 3H (Yu et al., 2017). This difference in phenotype indicated that *Rcr9* differed from $Rcr9^{ECD01}$. Another resistance gene, designated as $Rcr9^{wa}$, has also been identified from a turnip differential line in the ECD. It originated from cv.

'Waaslander' (ECD04), provided resistance to pathotype 5X, and was mapped into the same region as *Rcr9* (Karim et al., 2020). *Rcr9^{wa}* was mapped based on flanking markers into 12.3–12.6 Mb of chromosome A08 (slightly smaller interval than *Rcr9*). In addition, another resistance gene that originated from cv. 'Waaslander' and conferred resistance to pathotype 3H, designated as *Rcr3*, has been mapped into chromosome A08, flanked by SNP markers in position 11.3–11.6 Mb in the *B. rapa* 'Chiifu' reference genome v3.0 (Karim et al., 2020). The position of *Rcr3* was separated from *Rcr9^{ECD01}* (Figure 4). Also, gene *BraA.CR.b* for resistance to pathotype 3H was previously identified from the turnip differentials ECD01, ECD02, ECD03 and ECD04 and mapped into chromosome A08 (Hirani et al., 2018), but no information on the genome region corresponding to the *B. rapa* 'Chiifu' reference genome v3.0 was provided. Several genes for resistance to collections of *P. brassicae* from Japan and China, including *Crr1* (Suwabe et al., 2003), *CRs* (Laila et al., 2019), *PbBa8.1* (Chen et al., 2013) and *qBrCR38-2* (Zhu et al., 2019), have also been mapped into chromosome A08. The cloned CR gene *Crr1* was highly homologous to *Bra020861* in the *B. rapa* reference genome v1.5 and to *BraA08g014480* in the *B. rapa* reference genome v3.0, which is located in the *Rcr9^{ECD01}* genomic region. However, breeding lines carrying *Crr1* gene did not show resistance to the strains of *P. brassica* used in the current study (Yu, unpublished). Therefore, *Rcr9^{ECD01}* is unlikely the same as *Crr1*. The relationship of *Rcr9^{ECD01}* with *CRs* (Laila et al., 2019), *PbBa8.1* (Chen et al., 2013) and *qBrCR38-2* needs to be determined.

CRc was identified in Chinese cabbage cv. 'CR Kanko' derived from 'Debra', which was located into chromosome A02 (Sakamoto et al., 2008). However, this gene was not found in the DH population used for the current study.

Analysis of QTLs has been used for identification of several major genes for resistance to clubroot (Yu et al., 2017). A QTL that can be consistently detected with a PVE of >10% of trait value can be designated as the main effect QTL or major QTL (Wang et al., 2019). In this study, QTLs *Rcr10 ^{ECD01}* and *Rcr9^{ECD01}* were identified with 16.2 to 43.3% PVE. *Rcr10 ^{ECD01}* was identified based on the response to inoculation with pathotypes 3A, 3D and 3H. *Rcr9^{ECD01}* was identified based on the response to inoculation with all of the pathotypes used in this study. Therefore, both *Rcr10 ^{ECD01}* and *Rcr9^{ECD01}* appear to be major QTLs. The presence of the two major QTLs was also confirmed through BSA, which is consistent with the result obtained from the QTL analysis.

Clubroot severity in the DH lines in response to inoculation with the individual pathotypes was highly correlated, which indicated that resistance to these pathotypes was likely controlled by the same gene or tightly linked genes. However, the identification of QTLs in this study was based on relatively rough gene mapping, so it could not be determined if resistance to the pathotypes was controlled by a single gene or tightly linked genes. More detailed studies are in progress.

5) **Identification of CR genes other populations**

Similar to the methods described for ECD01/Debra, identification of CR genes was performed in other populations especially from turnip cv. 'Siloga' and breeding line 69-6992. Two DH populations Y193 and Y203 were developed from 'Siloga'. *RcrM* was identified in Y193, but not in Y203. Two CR genes, one on chromosome A08 at *Rcr9* locus, designated as *Rcr9^{Siloga}* and the other gene on chromosome A09 (not confirmed yet) were identified in Y203 (Table 1). Breeding lines with a cluster of genes on chromosome A08 (likely two genes) were developed in the population Y35 originating from *B. rapa* turnip 96-6992. Due to small size in the population, only line likely carrying a gene named *Rcr9^{6992}* (*Rcr9.3*) that could confer differential reactions was identified in the population Y35 (Table 1). The other line from Y35 likely carried two genes designated as *Rcr9.3+* was chosen from the population.

6) Selection of NILs from the DH population

After genetic mapping of CR genes from the populations list in Table 1, we could not identify *Crr1-Crr-4*, *CRc* and *CRk* described by the Japanese and Korean scientists using Canadian strains. Instead, four genes *Rcr3*, *Rcr9^{ECD01}* (*Rcr9.2*), *Rcr9^{6992}* (*Rcr9.3*) and *Rcr9^{Siloga}* (*Rcr9.4*) (Table 1) were identified on chromosome A08. Breeding lines carrying single genes respectively were chosen from the respective populations (Table 7).

Development of *Rcr3* lines was supported by another source of funding. Breeding lines carrying CR genes on

chromosome A03 (*Rcr1, RcrM* and *Rcr10*) were also developed. In addition, we developed DH lines carrying *Rcr8*, which was located on chromosome A02. The presence each of the genes was further genotyped with SNP markers associated with each of the genes through KASP analysis (Figure 9). In addition to the NILs carrying single genes, we selected three breeding lines carrying two CR genes (*Rcr9.2Rcr10*, *Avr9.3+* and *Avr9.4+*) each for further testing (Table 7).

7). Defining the pathogen population with the NILs

Due to the truly intracellular lifestyle of *P. brassicae*, clubroot is a complicated system to study and research of this plant pathogen system lacks somewhat behind other plant–pathogen relationships. So far, there are no any reports on identification of Avr genes interacted with specific CR genes. With the first NILs in brassica crops developed from the project, eight Avr genes corresponding to the eight CR genes in the NILs were proposed in *P. brassicae* based on gene for gene interaction (Figure 10).

The NILs were tested with 36 strains (Figure 11) collected in western Canada. Among the strains, 21, 13 and 2 were from Alberta, Saskatchewan and Manitoba respectively. Most of strains from Alberta were characterized with the CCD so the strains are named as 3A, 2B , etc. If there were more than one strains chosen from the same pathotype, a number was added after pathotype name so they could be differentiated. For example, there were three 3A strains tested in this study so the strains were named as 3A-1, 3A-2 and 3A-3. The strains from Alberta were all collected before 2018. The 13 strains from Saskatchewan designated as 'SK' were collected during 20018-2020 while the 2 strains from Manitoba were collected in 2018. The strains from Manitoba were provided by Pest Surveillance Initiative so the strains were names as 'PSI'.

As shown in Figure 12, differential reactions on the NILs were found with the strains tested. If a strain was avirulent to a NIL, the NIL showed resistant (R) response, which indicates an Avr gene corresponding the CR gene in the pathogen could be identified. The 36 strains were classified into 28 races (Figures 10 and 11) based the differential reactions. For example, the genotype for the race 1 strain was deduced as *AvrM-1-8.2-9.3-9.4* (Table 8). The most frequent race identified in this study was race 9 (*Avr8.2*) with 3 strains. Two strains each were identified in each of 6 races (races 8, 22, 23, 26, 27 28) . However, there was only 1 strain in each of 21 races (Table 8).

The frequency of *Avr* genes among the test strains was in a range of 16.7% to 58.3% (Figure 13), indicating no single genes could effectively control all the races, but their effectiveness was varied with genes. The presence of *Avr9.4* was 58.3%, which was the most frequent *Avr* gene in this study. This *Avr* gene was proposed to interact with $Rcr9^{Siloga}$(*Rcr9.4*). Therefore, $Rcr9^{Siloga}$ could be considered the most effective gene for controlling clubroot.

RcrM is the CR gene present in the first generation of Canadian CR cultivars. The frequency of *AvrM* among the strains was 22.2%, indicating that the cultivars may not be very effective for controlling clubroot disease in western Canada anymore. Two CR genes $Rcr9^{ECD01}$ (*Rcr9.2*) and $Rcr10^{ECD01}$ were identified in turnip ECD01. The presence of *Avr9.2* and *Avr10* corresponding the CR genes was at the frequency of 19.4% and 36.1% respectively. Interestingly, effectiveness for controlling clubroot was 97.2% when the two CR genes worked together. Similarly, breeding lines with genes $Rcr9^{6992}$ (*Rcr9.3*) and $Rcr9^{Siloga}$ (*Rcr9.4*) plus additional genes corresponding to *Avr9.3+* and *Ave9.4+* in the pathogen were resistant to the strains tested at 97.2% and 94.4% respectively (Figure 13). Therefore, pyramiding CR genes is very important for the disease control.

10. Conclusions and Recommendations: *Highlight significant conclusions based on the findings of this project, with emphasis on the project objectives specified above. Provide recommendations for the application and adoption of the project findings. (**Maximum of 500 words**)*

We have developed the set of NILs containing single CR genes based on our current knowledge on genetic mapping for resistance to Canadian strains of *P. brassice* using the conventional breeding methods, microspore culture and marker assisted selection. Despite lots of further studies needed, these lines could replace the current CCD set for identifying races of *P. brassicae*. In addition, this set of canola lines consisting of eight single genes could be used for monitoring changes in the pathogen populations in canola fields. Theoretically, the set of lines has ability to define 2^8= 256 races. Such a system will show which resistance genes are currently

effective against the pathogen, identify isolates of the pathogen that can discriminate among the resistance genes in registered cultivars, and provide opportunities for use of deployment strategies for resistance genes that will increase their durability for the future. This will be used for collecting highly informative surveys of the pathogen population and provide information concerning the effectiveness of resistance.
The NILs can be used for genetic studies on the pathogen such as identification and cloning of the potential Avr genes, developing SNP markers associated with each of the Avr genes. A new direction of great significance to the canola industry can be established in the near future.
We have developed canola DH lines carrying more than one CR genes with resistance introgressed from three sources (ECD01, Siloga and 6992) of *B. rapa* turnips. These lines showed high level of resistance to the majority of Canadian races of the clubroot pathogen identified in this study. In addition to the importance for canola breeding programs, these valuable materials open opportunities for studies on CR gene pyramiding and gene interactions, facilitating development of more robust strategies for control of the disease.
More than 1200 DH lines have been developed in the project. As the pathogen evolves, new emerging races of the pathogen can be identified in the canola fields on the Prairie. The DH populations can serve as the ideal materials for identification of novel resistant genes.

11. Is there a need to conduct follow up research? *Detail any further research, development and/or communication needs arising from this project*.

Due to the urgent need in Canadian canola industry, we have been working very hard to develop the set of NILs that should be better than the current CCD set for differentiating Canadian *P. brassicae* strains. However, there is a need to conduct more studies to validate the NILs, improve their differential power by adding more NILs or replacing some NILs with better lines. For race profiling, only small number of stains were tested so more studies on this should be initiated.

1) Validate the NILs by fine mapping of the CR genes: Developing the NILs largely relied on genetic mapping of CR genes. Eight single genes were used for developing the NILs. Except *Rcr1*, fine mapping of the rest of genes has not been performed. Up to now, four clusters of CR genes, two on chromosome A03, one on A02 and one on A08 for resistance to Canadian strains of clubroot have been identified in Dr. Fengqun Yu's group at AAFC. The relationship of the CR genes in the NILs with the clusters are to be determined.
2) Improve their differential power by identify more CR genes: As shown in Table 1, approximately 1300 DH were developed from the project. It is possible to identify more CR genes in the DH populations. Once the additional genes are identified, more NILs can be added into the existing NILs set. Differential power will be increased accordingly. For example, if two more NILs are added, the set can differentiate 2^{10} = 1076 races of the pathogen.
3) Replace some NILs with better lines: It was very challenging for developing the NILs. Originally, the project focused on breeding. As several requirements for this project such as identified CR genes, differential isolates and molecular markers for selection were not available, the group had to conduct studies on both genetics and breeding. Selection in the greenhouse and the field conditions were conducted. All the NILs are spring type so vernalization is not required. However, only a small field trial was performed so the lines may not be the best selection. Further evaluating resistant DH lines in the DH populations in field conditions will help to assess the lines agronomical traits more thoroughly so better NILs could be possibly obtained.
4) Capture a big picture of race structure through a large-scale survey: The race structure determined in the current study was based on 36 strains. A survey in different geographical regions with multiple years is needed.
5) Only small amount of seeds each line have been produced. A field trial for increasing seeds will be performed in 2022.

12. Patents/ IP generated/ commercialized products: *List any products developed from this research.*

1) Near isogenic lines carrying five CR genes (*Rcr1, RcrM(s), Rcr8, Rcr9^{ECD01} and Rcr10^{ECD01}*) were delivered into the CCII members in April, 2022.
2) A Plant variety/germplasm disclosure form for four clubroot resistant lines introgressed from turnips (ECD01 and 96-6992) was submitted in August 2021.
3) Three SNP markers linked to *Rcr9^{ECD01}* on chromosome A08, 3 to *Rcr10^{ECD01}* on chromosome A03 developed from an BC2DH population and 11 on chromosome A08 from other populations for KASP assay were distributed to the second AAFC Clubroot Consortium members in Feb 2022.
4) Two breeding lines carrying genes *Rcr1* and *Rcr3* respectively have been transferred to the mustard breeder at AAFC for developing *B. juncea* for resistance to clubroot in December 2021.
5) Information on genetic mapping of *Rcr9^{ECD01}* and *Rcr10^{ECD01}* and SNP markers linked to the genes were distributed to the second AAFC Clubroot Consortium members in August 2021.
6) *B. napus* breeding lines with introgressed clubroot resistance genes from turnip to all Canadian pathotypes of *P. brassicae* were distributed to the second AAFC Clubroot Consortium members in April 2021.
7) A high efficient method for screening plants for resistance to clubroot was transferred into the members of AAFC Clubroot Consortium II in December 2019

13. List technology transfer activities: *Include presentations to conferences, producer groups or articles published in science journals or other magazines*.

Oral presentations

1) Genetics and breeding for resistance to clubroot. Virtual, Saskatchewan Clubroot Initiative Committee meeting, February 2022
2) Defining populations of *Plasmodiophora brassicae* with near isogenic *Brassica napus* lines. WCC/RRC Pathology Sub Committee Meeting, Saskatoon, February 2021
3) Developing *Brassica napus* near isogenic lines for resistance to clubroot. WCC/RRC Pathology Sub Committee Meeting, Saskatoon, February 2021
4) Genetic resistance to canola diseases - a case study with developing genetic resources for resistance to clubroot. Saskatoon Research and Development Centre, AAFC, December 2020
5) Resistant sources and resources for clubroot at AAFC, Saskatoon. Clubroot Steering Committee Meeting, Saskatoon, April 2020
6) Developing spring type *Brassica napus* near isogenic lines for differentiating races of *Plasmodiophora brassicae*. WCC/RRC Pathology Sub Committee Meeting, Saskatoon, February 2020
7) Control of canola diseases using genetic resources from its relatives. Northwest A&F University, Yangling, Shaanxi, China, November 2018 (Third party paid travel, 2 hours lecturer)
8) Developing spring type *Brassica napus* lines containing single clubroot resistance genes. International Clubroot Workshop. Edmonton, August 2018 (Plenary speaker)

Publication

Yu F, Zhang Y, Wang J, Chen Q, Karim M, Gossen BD, Peng G (2022) Identification of two major QTLs in *Brasica napus* lines with introgressed clubroot resistance from turnip cultivar ECD01. **Front. Plant Sci**. https://doi.org/10.3389/fpls.2021.785989

14. List any industry contributions or support received.

Development of the NIL line carrying *Rcr3* was under the AAFC Clubroot Consortium, which was funded by Bayer (BASF), Cargill, Cibus, Corteva, CPS (Nutrien), DL seeds and Monsanto (Bayer).

15. Acknowledgements. *Include actions taken to acknowledge support by the Ministry of Agriculture and the Canada-Saskatchewan Growing Forward 2 bilateral agreement (for projects approved during 2013-2017) or Canadian Agriculture Partnership (For projects approved beyond 2017).*

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16. Appendices: *Include any additional materials supporting the previous sections, e.g. detailed data tables, maps, graphs, specifications, literature cited (Use a consistent reference style throughout).*

Tables

Table 1. Developing DH lines carrying clubroot resistance genes identified by Japanese and Korean scientists or novel genes for resistance to Canadian strains of *P. brassicae*.

Code Name	No. of DH lines	Donor	Generation	pathotype	Selection for CR genes	Confirmed CR genes*
Y30	20	Mendel	BC2	3H	*RcrM*	*RcrM*
Y193	199	Siloga	BC3	3H	*Crr1, Crr2 and Crr3*	*RcrM*
Y203	118	Siloga	BC3	5X-LG2	*Crr1, Crr2 and Crr3*	$Rcr9^{Siloga}$ and a gene A09
Y194	99	ECD01/Debra	BC3	5X-LG2	*CRc,CRk, Crr3*	*TBD*
Y358	18	ECD01/Debra	BC4	LG2	*CRc,CRk, Crr3*	*TBD*
Y154	84	ECD01/Debra	BC2	3H	*CRc,CRk, Crr3*	$Rcr9^{ECD01}$, $Rcr10^{ECD01}$
Y294	162	ECD01/Debra	BC3	3H	*CRc,CRk, Crr3*	*TBD*
Y309	12	ECD01/Debra	BC3	3H	*CRc,CRk, Crr3*	*TBD*
Y310	202	Milan	BC3	3H	*Crr3*	All susceptible to 3H. Crr1 is no present in the population.
Y399	140	ECD02	BC3	5X-LG2	*CRa*	$Rcr9^{ECD02}$ in the population is the same as $Rcr9^{ECD01}$.
Y450	43	ECD02	BC3	3H	*CRa*	$Rcr9^{ECD02}$ in the population is the same as $Rcr9^{ECD01}$.
Y529	87	FN	BC2	5X-LG1	*unknown*	$Rcr9^{FN}$, but the same as $Rcr9^{Siloga}$
Y35	60	69-6992	BC2	3H	*unknown*	$Rcr9^{6992}$ and additional gene(s) on A08
Y1020	15	T19	BC3	5X-LG2	*Rcr8*	*Rcr8*
Total	1259					

*Six DH lines with single CR genes (in red) were chosen from the respective populations. The *Rcr1* line was in BC4S2, which is not listed in the Table. The *Rcr3* line was developed using other source of finding so it is not presented in the report.

Table 2. Molecular cloning of clubroot resistance genes at *Rcr1* locus

Cultivar	CR gene	Phenotype 3	Genotype (similarity to *CRa*)	Group
FN	*Rcr1*	R	100%	I

JNC	*Rcr2*	R	100%	I
ECD02	*CRa*	R	100%	I
Mendel	*RcrM*	R	88.30%	II
ACDC		S	85.50%	III

Table 3. Genetic analysis of resistance of the parental lines (DH16516, ECD01), controls (cv '45H29') and the BC_2 DH population inoculated with four pathotypes of *Plasmodiophora brassicae* based on the clubroot severity (disease severity index, DSI) of each line (Resistant, R, DSI ≤ 30; Susceptible, S, DSI > 30).

Pathotype	DSIs				No. of DH lines			P-value of ratio		
	ECD01	DH16516	F_1	45H29	Total	R	S	1:1	3:1	1:3
3A	0	100	-	100	82	27	55	0.001	0.001	0.100
3D	0	100	-	100	80	49	31	0.001	0.005	0.001
3H	0	100	0	0	82	61	21	0.001	0.90	0.001
5X	0	100	-	100	84	52	32	0.001	0.006	0.001

Table 4. Correlation coefficients for clubroot severity after inoculation of DH population derived from BC_2 of DH16516 × ECD01 for resistance to four pathotypes of *Plasmodiophora brassicae*. **Significance level at $P < 0.01$.

Pathotype	3A	3D	3H	5X
3A	1.00			
3D	0.64**	1.00		
3H	0.65**	0.81**	1.00	
5X	0.55**	0.55**	0.68**	1.00

Table 5. QTL position, phenotypic variation explained (PVE), additive (Add), logarithm of the odds (LOD) and confidence interval (CI) for the QTLs originating from *Brassica rapa* ECD01 for resistance to four pathotypes of *Plasmodiophora brassicae* (permutations = 1000).

Path-otype	Chromosome / QTL	Posi-tion	Left Marker	Right Marker	LOD	PVE (%)	Add	Left CI	Right CI
3A	A03 / *Rcr10*^{ECD01}	0	DM_A03_ 12570715	DM_A03_ 10873502	5.6	16.2	14.3	0.0	1.5
	A08 / *Rcr9*^{ECD01}	38	DM_A08_ 10325589	DM_A08_ 10529713	11.4	43.3	22.4	35.5	42.5
3D	A03 / *Rcr10*^{ECD01}	0	DM_A03_ 12570715	DM_A03_ 10873502	7.2	27.3	21.4	0.0	2.5
	A08 / *Rcr9*^{ECD01}	38	DM_A08_ 10325589	DM_A08_ 10529713	5.2	21.5	18.1	32.5	44.5

3H	A03 / *Rcr10*^{ECD01}	0	DM_A03_ 12570715	DM_A03_ 10873502	6.2	17.9	16.6	0	2.5
	A08 / *Rcr9*^{ECD01}	35	DM_A08_ 10337601	DM_A08_ 10325589	12.2	43.1	25.2	33.5	37.5
5X	A08 / *Rcr9*^{ECD01}	34	DM_A08_ 10337601	DM_A08_ 10325589	10.9	42.8	25.4	32.5	37.5

Table 6. A list of genes encoding proteins associated with plant disease resistance through BLAST2GO and Blast searches with CDS in the QTL target regions at https://www.arabidopsis.org/Blast/index.jsp.

QTL	***Rcr10***^{ECD01}	***Rcr3/9***^{ECD01}	
R to pathotype	3A, 3D and 3H	3A, 3D, 3H and 5X	
Chromosome	A03	A08	
Gene name	*BnaA03g25330D*	*BnaA08g10100D*	*BnaA08g11840D*
***B. napus* gene location (base)**	12234711…12240552	9456084…9467947	10622229…10625339
Length (base)	5841	11863	3110
Description from Blast2Go	Disease resistance protein RPS6-like	Disease resistance protein TAO1-like	Probable disease resistance protein At4g33300
Function with Blast2Go	Hydrolase activity; ADP binding; defense response; signal transduction	Hydrolase activity; ADP binding; defense response; signal transduction	ADP binding
Homolog in Arabidopsis	*AT5G22690*	*AT5G11250*	*AT4G33300*
R gene class	Disease resistance protein (TIR-NBS-LRR class) family	TIR-NBS-LRR protein involved in stress response	Activated disease resistance 1 (ADR1) family of NBS-LRR immune receptors

Table 7. Near isogenic lines chosen from more than 1000 DH lines shown in Table 1

R Donor	**Population**	**Breeding methods**	**CR gene**	**CR label suggested**	**Days to flowering (Saskatoon 2020)**
T19	Y1020	Microspore culture	*Rcr8*	*Rcr8.2*	48
FN	Y549	Conventional breeding	*Rcr1*	*Rcr1*	48
Mendel/Siloga	Y30 and Y193	Microspore culture	*RcrM*	*RcrM*	52
ECD01 (Debra)	Y154	Microspore culture	*Rcr10*^{ECD01}	*Rcr10*	52
*Mendel	MR1510	Microspore culture	*Rcr3*^{Mendel}	*Rcr3*	NA

ECD01 (Debra)	Y154	Microspore culture	*Rcr9^{ECD01}*	*Rcr9.2*	52
96-6992	Y35	Microspore culture	*Rcr9^{6992}*	*Rcr9.3*	52
Siloga	Y203	Microspore culture	*Rcr9^{Siloga}*	*Rcr9.4*	52
ECD01 (Debra)	Y154	Microspore culture	*Rcr9^{ECD01}* *Rcr10^{ECD01}*	two genes *Rcr9.2 Rcr10*	52
96-6992	Y35	Microspore culture	*Rcr9^{6992}* and additional gene(s) on A08	More than one genes *Rcr9.3+*	52
Siloga	Y203	Microspore culture	*Rcr9^{Siloga}* and a gene A09	More than one genes *Rcr9.4+*	55
DH16516					49-52

*A second gene was identified from the European canola cultivar 'Mendel', which was funded by another source of funding.

Table 8. Race structure of the 36 strains collected in western Canada

Race#	Race genotype	No. of isolates
1	AvrM-1-8.2-9.3-9.4	1
2	Avr8.2-9.2-9.3-10	1
3	Avr3--8.2-9.2-9.3	1
4	Avr3-8.2-9.2	1
5	Avr3-8.2	1
6	Avr8.2-9.2-9.3	1
7	Avr8.2-9.3-9.4	1
8	Avr8.2-9.3	2
9	Avr8.2	3
10	AvrM-1-9.2-9.4-10	1
11	Avr1M-1-9.3-9.4	1
12	AvrM-1-9.4	1
13	Avr1-9.4-10	1
14	Avr1-9.3-9.4	1
15	AvrM-3-9.3-9.4-10	1
16	AvrM-3-9.4-10	1
17	AvrM-9.3-9.4-10	1
18	AvrM-3-9.3-9.4	1
19	Avr3-9.2-9.3-9.4-10	1
20	Avr3-9.3-9.4-10	1
21	Avr9.2-9.3-9.4-10	1
22	Avr9.3-9.4-10	2

23	Avr9.4-10	2
24	Avr3-9.3-9.4	1
25	Avr3-9.3	1
26	Avr9.3-9.4	2
27	Avr9.3	2
28	No Avr	2

Figures

Figure 1. Producing homozygous plants through microspore culture

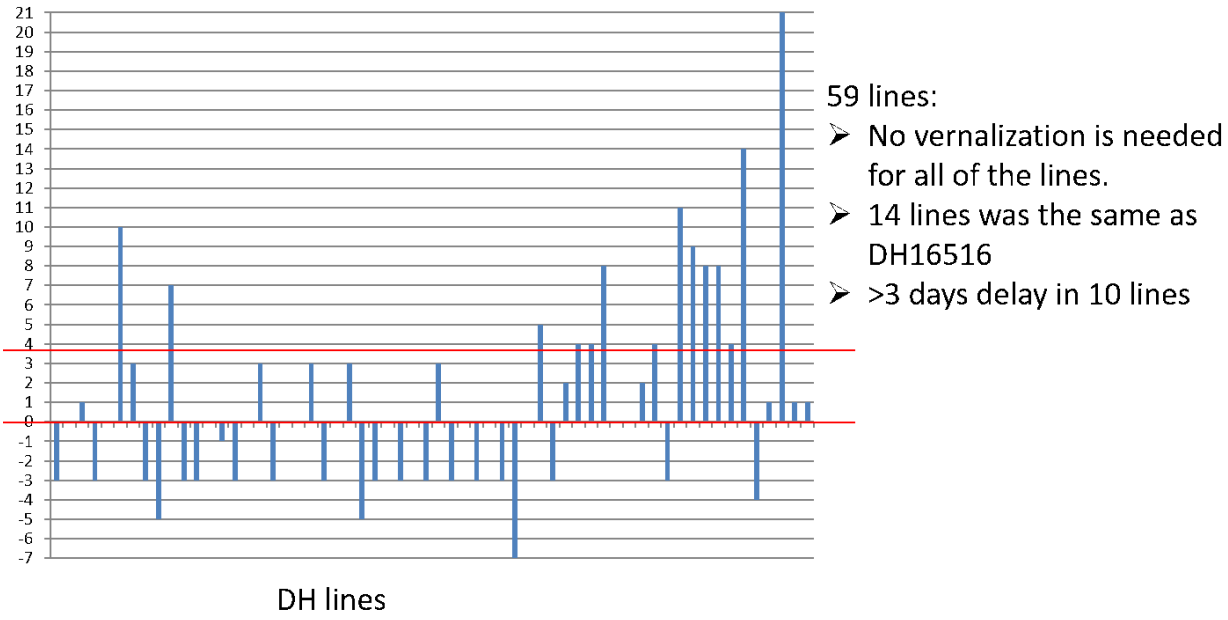

Figure 2. Days to flowering comparing with the recipient B. napus line DH16516 from DH population originating ECD01/Debra

Figure 3. Field trial in Saskatoon 2020: A. Five weeks after seeding; B. Plants with isolating tents; C. Mature plants for harvesting.

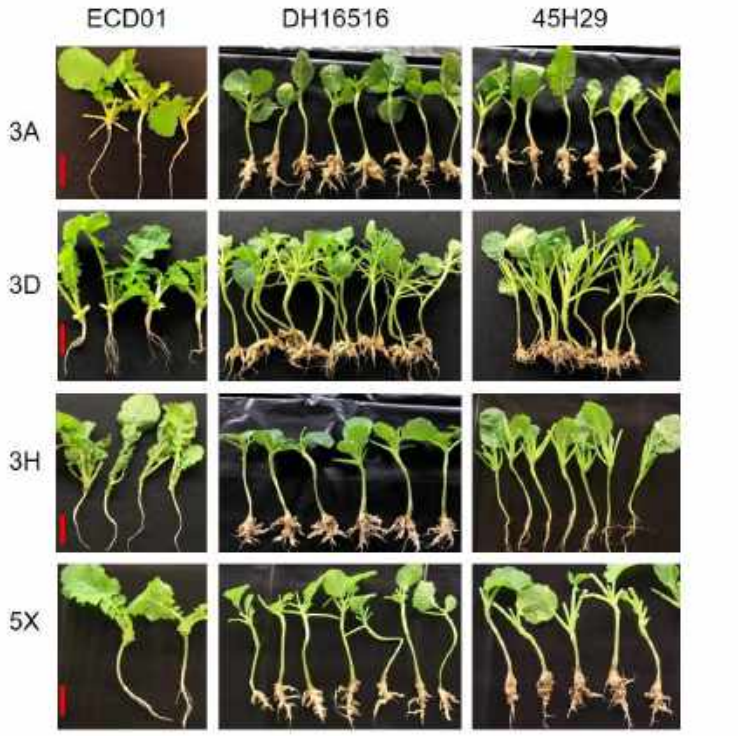

Figure 4. Plant phenotypes and clubroot response at five weeks after inoculation in the parental lines (Debra and DH16516) and a control cultivar (45H29) to inoculation with four pathotypes (3A, 3D, 3H and 5X) of Plasmodiophora brassicae under controlled conditions. The bars represent 5 cm in length.

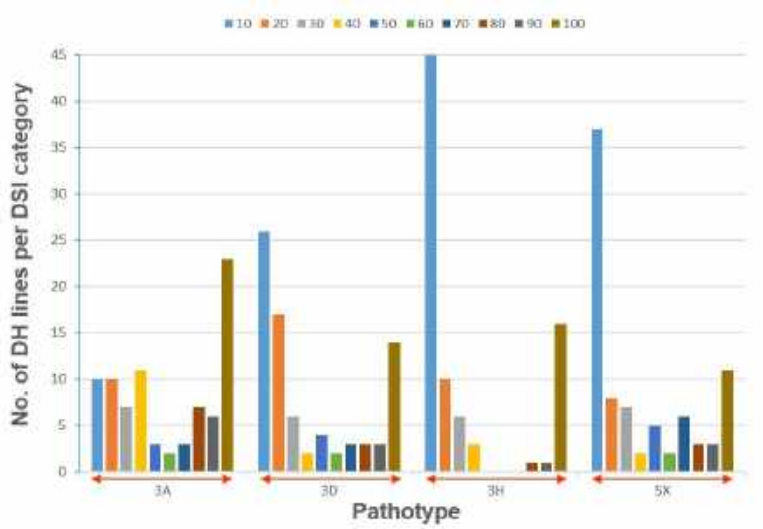

Figure 5. Distribution of clubroot severity (disease severity index, DSI) following inoculation with four pathotypes (3A, 3D, 3H and 5X) of *Plasmodiophora brassicae* in a DH population derived from a BC_2 plant of *Brassica rapa* ECD01 crossed with *Brassica napus* DH16516. Colours in each stacked column represent the proportion of the lines with a DSI value within that decile (= 10% range).

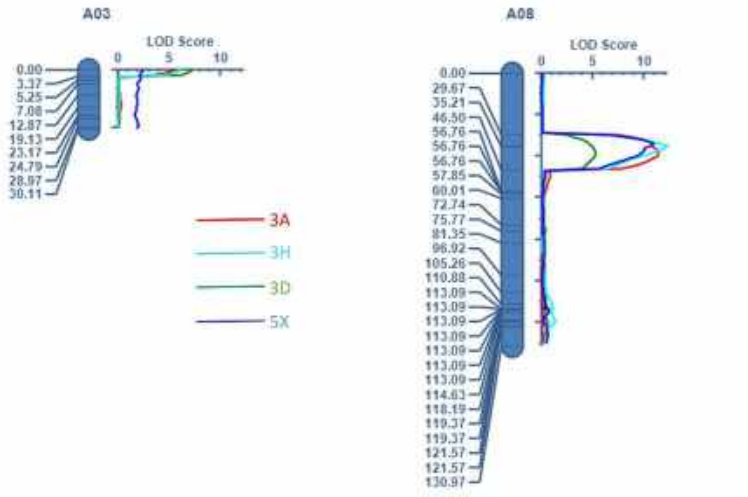

Figure 6. **Two QTLs detected: *Rcr10*^{ECD01} on chromosome A03 and *Rcr9*^{ECD01} on A08.**

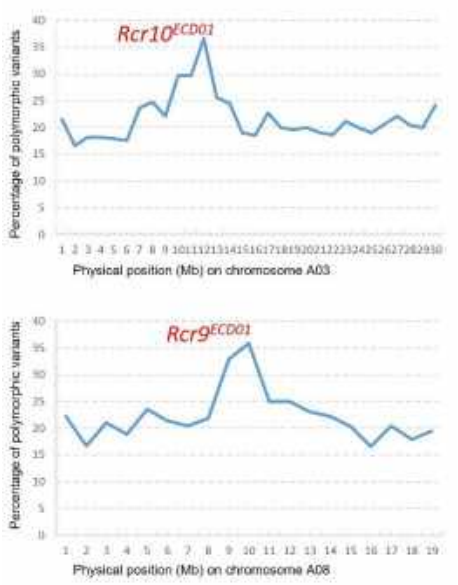

Figure 7. Distribution of polymorphic variants (%): One peak for Rcr10 ^{ECD01} on chromosome A03 and the other for Rcr9^{ECD01} on A08 were identified through bulk segregant analysis with the mapping method of the percentage of polymorphic variants described by Yu et al (2016) and Dakouri et al. (2018).

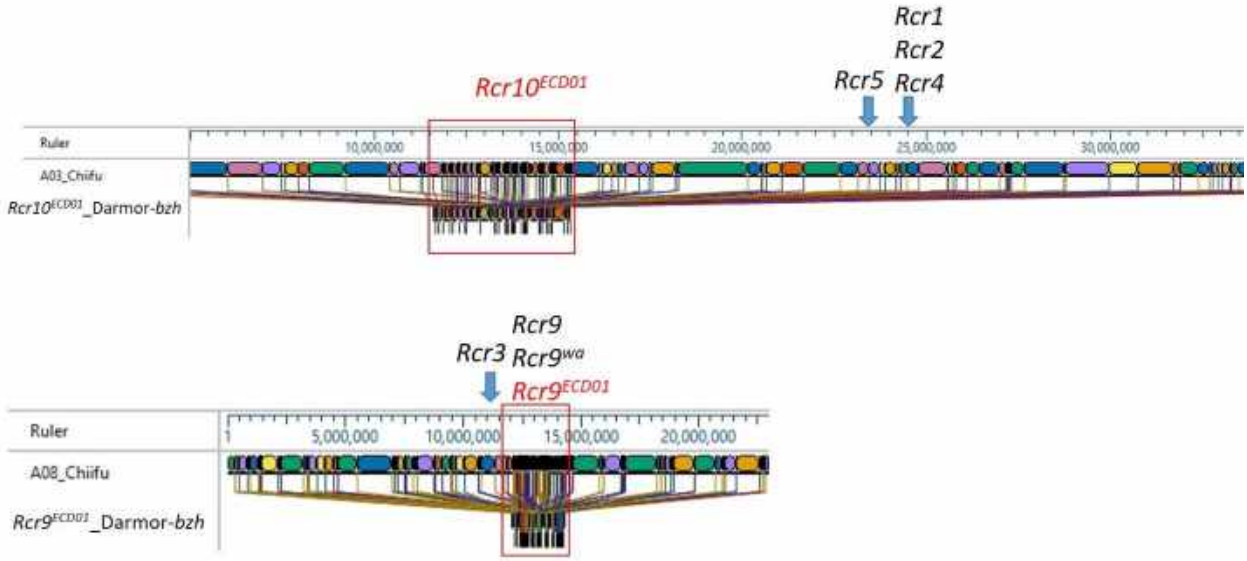

Figure 8. Maps of the *Rcr10^{ECD01}* and *Rcr9^{ECD01}* target regions in A03 and A08 of Brassica napus 'Darmor' and the homologous regions of the B. rapa reference genome 'Chiifu' v3.0.

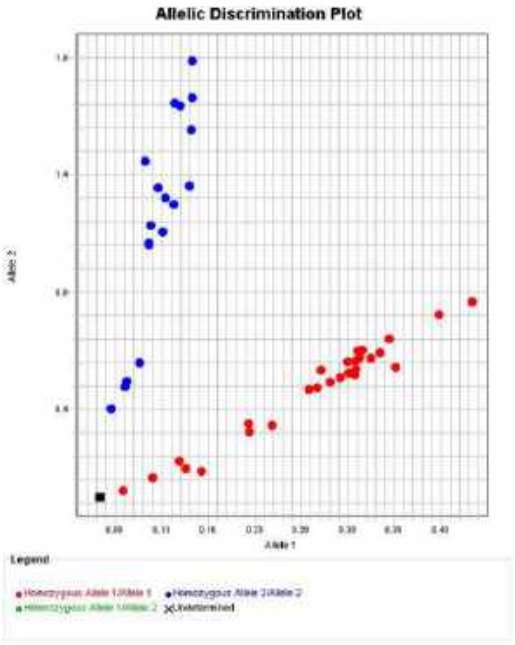

Figure 9. Confirming the presence of *Rcr9*^{ECD01} in the selected plants in the population Y154: Allele 1 from R parent ECD01 and allele 2 from S parent DH16516. Individuals from the red cluster were resistant while those from the blue cluster were susceptible.

R genes and Avr genes

Brassica napus			***Plasmodiophora brassicae***
A03	*RcrM*	← - - - - - - - - - - →	*AvrM*
A03	*Rcr1*	← - - - - - - - - - - →	*Avr1*
A08	*Rcr3*	← - - - - - - - - - - →	*Avr3*
A02	*Rcr8.2*	← - - - - - - - - - - →	*Avr8.2*
A08	*Rcr9.2* (ECD01)	← - - - - - - - - - - →	*Avr9.2*
A08	*Rcr9.3*	← - - - - - - - - - - →	*Avr9.3*
A08	*Rcr9.4*	← - - - - - - - - - - →	*Avr9.4*
A03	*Rcr10* (ECD01)	← - - - - - - - - - - →	*Avr10*

Figure 10. Hypothetical gene for gene interaction of *B. napus* (canola) and *P. brassicae*

Race #	No. of strains	Name	Race #	No. of strains	Name
1	1	PSI11	15	1	SK25
2	1	3O	16	1	SK21
3	1	8J	17	1	3D-2
4	1	SK10	18	1	SK27
5	1	SK29	19	1	SK16
6	1	5X-LG-1	20	1	8P
7	1	PSI10	21	1	SK04
8	2	5X-LG-3; SK24	22	2	8E-1; 3D-3
9	3	5K; 5X-LG2; SK20	23	2	3A-3; 3D-1-SP
10	1	SK18	24	1	SK22
11	1	5G-1	25	1	3A-2
12	1	3H	26	2	3A-1; SK28
13	1	EXP-16-4	27	2	2B-1; 3D-1
14	1	5L-1	28	2	5C; SK26

PSI: Pest Surveillance Initiative, MB

Figure 11. 36 strains of *P. brassicae* were classified into 26 races**.**

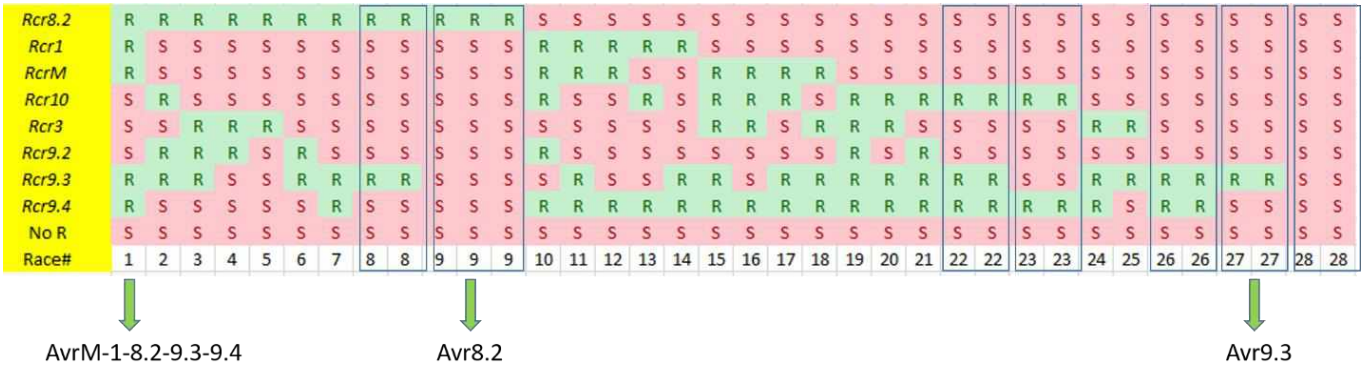

Figure 12. Differential reactions among the near isogenic lines with strains collected in western Canada

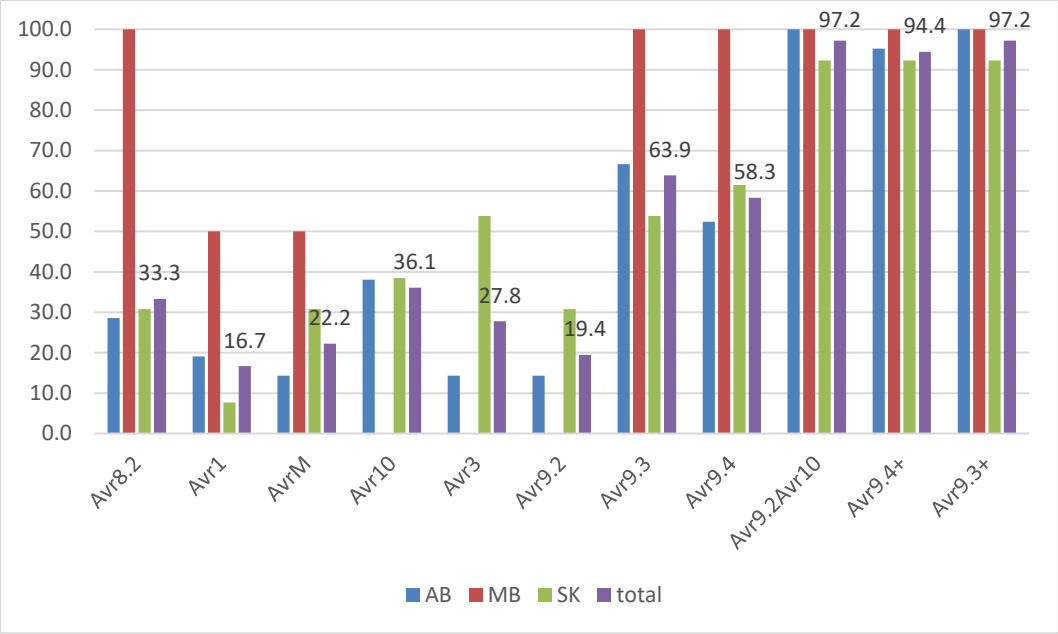

Figure 13. Avr profiling of *P. brassicae* in western Canada

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