Identification and genetic mapping of resistance genes against Canadian pathotypes of *Plasmodiophora brassicae* in *Brassica rapa* and *Brassica napus*

by

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Abstract

Clubroot (*Plasmodiophora brassicae*) is an important disease of canola/rapeseed (*Brassica napus*) and other crucifers. In this study, resistance loci/genes effective against *P. brassicae* pathotypes 2B, 3A, 3D, 3H, 5C, 5X and 8J were identified and mapped via genotyping-by-sequencing, QTL analysis, conventional linkage mapping, and by exploring the effect of individual genes on quantitative traits in combination with marker and trait data. Four of 34 *B. napus* accessions, CGN06902, CGN17369, AAFC695, and CN46235, were resistant to pathotypes 3A, 2B, and 3D, while 16 *B. rapa* families did not show any resistance. The resistant accession AAFC695, *B. napus* 'Mendel' (resistant to 13 of 17 pathotypes identified on the Canadian Clubroot Differential set), and *B. rapa* line ECD02 (resistant to all 17 pathotypes) were used to map resistance genes by developing a BC_1S_1 population based on ECD02 and doubled haploid (DH) populations based on 'AAFC695' and 'Mendel'. A single QTL $Rcr9^{ECD02}$ was identified on chromosome A08 of ECD02 using 93 BC_1S_1 lines; this QTL conferred resistance to pathotypes 3A, 3D, 3H, and 5X, accounting for 68.9-74.4% phenotypic variation explained (PVE). Bulked segregant and KASP analysis confirmed the QTL. In 'AAFC695', a QTL $Rcr9^{AAFC695}$ was identified on chromosome A08 using 102 DH lines; $Rcr9^{AAFC695}$ explained 92.6-95.0% PVE in response to pathotypes 3A, 3D, 3H, and 5X, with LOD values of 60.3 to 70.8. In 'Mendel', the QTL $Rcr3^{Mendel}$ was mapped on chromosome A08 using 137 DH lines; this was a major locus explaining 88-90% PVE for resistance to pathotypes 3D, 5C, and 8J, with an additive effect of 47.2-47.3%. Testing of 60 lines from this population against pathotype 3H indicated that $Rcr3^{Mendel}$ is the *Rcr3* locus, but that it also bestows resistance to pathotypes 3D, 5C, and 8J. The identification of TIR_NBS_LRR (TNL) genes (via analysis of gene annotation with Blast2GO and gene ontology information) in the QTL regions of the three populations

confirmed that the identified QTL *Rcr9*^{ECD02}, *Rcr9*^{AAFC695} and *Rcr3*^{Mendel} are different from each other, and distinct from *Rcr9*. The CR loci/genes identified in this study will enrich the repository of resistance genes available to protect canola/rapeseed from clubroot disease.

Preface

Md Mizanur Rahaman submitted this dissertation in partial fulfillment of the requirements for the degree Doctor of Philosophy in Plant Sciences. He conducted all of the experiments, collected and analyzed data, and prepared the first drafts of each chapter.

Chapter 3. A version of the chapter has been published in *The Plant Genome* as Rahaman, M., Strelkov, S.E., Hu, H., Gossen, B.D., & Yu, F. 2022. Identification of a genomic region containing genes involved in resistance to four pathotypes of *Plasmodiophora brassicae* in *Brassica rapa* turnip ECD02. The Plant Genome e20245. https://doi.org/10.1002/tpg2.20245

Md Mizanur Rahaman conducted the experiments, collected and analyzed data, and prepared the draft manuscript. Hao Hu helped with the KASP analysis. Drs. Bruce Gossen, Stephen Strelkov, and Fengqun Yu reviewed and edited the manuscript. Dr. Fengqun Yu provided project guidance and served as the corresponding author on the submitted manuscript.

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Md Mizanur Rahaman conducted the experiments, collected and analyzed data, and wrote the draft manuscript. Hao Hu helped with KASP analysis. Dr. Fengqun Yu supervised the experiments, reviewed and edited the draft version of the manuscript, and helped interpret the results. Drs. Strelkov and Gossen reviewed and edited the manuscript prior to submission.

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Md Mizanur Rahaman conducted the experiments, collected and analyzed data, and prepared the draft manuscript. Hao Hu and Masud Karim helped with KASP analysis. Dr. Fengqun Yu supervised the experiments, reviewed and edited the draft version of the manuscript, and helped interpret the results. Drs. Strelkov and Gossen reviewed and edited the manuscript prior to submission.

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List of Abbreviations

AAFC	Agriculture and Agri-Food Canada
Add.	Additive effect
BC1	First backcross generation
BLAST	Basic local alignment
BRAD	Brassica Database
BSA	Bulked-segregant analysis
CCD	Canadian Clubroot Differential
CR	Clubroot-resistant
dai	Days after inoculation
DH	Doubled haploid
DSI	Disease severity index
ECD	European Clubroot Differential
ETI	Effector-triggered immunity
F1	First-generation
GBS	Genotyping-by-sequencing
ICIM	Inclusive composite interval mapping
JA	Jasmonic acid
KASP	Kompetitive allele-specific PCR
LOD	The logarithm of the odds
LRR	Leucine-rich repeats
MAMPs	Microbe-associated molecular patterns
Mb	Million base pair
NBS	Nucleotide-binding site
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PR	Pathogenesis-related
PRRs	Pattern recognition receptors
PTI	Pattern-triggered immunity
PVE	Phenotypic variation explained

QTL	Quantitative trait locus
RIL	Recombinant inbred line
ROS	Reactive oxygen species
RT-PCR	Real-time PCR
SA	Salicylic Acid
SCAR	Sequence characterized amplified region
SD	Segregation distortion
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeat
TAIR	The Arabidopsis Information Resource
TIR	Toll-interleukin-1
TNL	TIR-NBS-LRR
WGRS	Whole-genome resequencing

Chapter 1. Introduction and literature review

1. 1 General introduction

The Brassicas include many economically important oilseed and vegetable crops (Dixon 2009). Historically, six Brassica species (Morinaga 1934; cited by Raymer et al. 2002) have been cultivated in different parts of the world as oilseeds, forages, condiments, or vegetables. These include *Brassica juncea* L. (Indian or brown mustard), *B. carinata* L. (Abyssinian or Ethiopian mustard), *B. napus* L. (Swede rape or oilseed rape), *B. nigra* L. (black mustard), *B. rapa* L. (turnip rape, turnip, Chinese cabbage) and *B. oleracea* L. (cabbage, cauliflower, broccoli, kale, Brussel sprouts, etc.). The vegetable Brassicas provide significant health benefits, helping to reduce the risk of cancer, heart disease, and strokes (Manchali et al. 2012). Similarly, the oil extracted from oilseed Brassicas is one of the major sources of "heart-healthy" unsaturated fatty acids, while saturated fatty acids from these species are useful for industrial applications (Dixon 2007). *Brassica napus* (oilseed rape or canola) is one of the major oil-producing species and represents the second most widely grown edible oil crop in the world after soybean (Kupiec et. al. 2020; USDA 2020).

Factors that can limit canola yield and quality include diseases and insects, as well as abiotic factors associated with environmental conditions or crop management practices (Canola Council of Canada 2019). Clubroot, a damaging soilborne disease of crucifers caused by the obligate parasite *Plasmodiophora brassicae* Wor., occurs in most of the Brassica-growing regions worldwide (Dixon 2009), and is a major concern for canola production in Canada; this crop contributes an estimated $15.4 billion annually to the national economy (Rempel et al. 2014; Canola Council of Canada 2020). Infection by the clubroot pathogen can result in yield

losses of 30%–100% under conditions favorable for the disease (Strelkov et al. 2007; Tewari et al. 2005). Since the first identification of clubroot on canola in the Canadian Prairies in 2003, the disease has spread rapidly, with more than 3,300 field infestations confirmed in Alberta by 2019 (Strelkov et al. 2020). Clubroot is also a concern for canola production across the rest of the Canadian Prairies (Cao et al. 2009; Dokken-Bouchard et al. 2010; Hollman et al. 2021; Strelkov et al. 2012) and the Northern Great Plains of the United States (Chapara et al. 2019; Chittem et al. 2014).

The life cycle of *P. brassicae* and related species includes the production of zoospores, multinucleate plasmodia, and long-lived resting spores (Braselton 1995). In addition to *B. napus*, *P. brassicae* can attack many other hosts in the Brassicaceae family (Karling 1968; cited by Dixon 2009). Infection by *P. brassicae* is associated with the abnormal swelling of the host root tissue, which is a characteristic symptom of clubroot and results in the formation of large root galls. Severe clubbing of the roots inhibits water and nutrient uptake from the soil, leading to stunting, wilting, and premature death of infected plants (Strelkov et al. 2007). The resting spores are the primary inoculum of *P. brassicae*. Under high moisture conditions, the resting spores germinate to produce primary zoospores, with germination rates enhanced by the presence of root exudates (Rashid et al. 2013). The motile zoospores attach to and encyst on the root hairs, then penetrate to form a primary plasmodium inside the root hair (Kageyama and Asano 2009). The primary plasmodia mature to produce and release secondary zoospores, which penetrate the root cortical tissues to produce secondary plasmodia. Secondary infection and the physiological and morphological changes associated with this process result in the development of characteristic galling of the roots (Dixon et al. 2013). Eventually, the secondary plasmodia cleave into a new generation of resting spores, which are released back into the soil as the

clubbed roots decompose (Moxham et al. 1983). These spores can survive in the soil for many years (Gossen et al. 2014; Hwang et al. 2019; Karling 1968; Peng et al. 2014; Strelkov et al. 2007; Wallenhammar 1996), and act as an inoculum source to infect the next available crop.

The management of clubroot is a challenge for successful canola/Brassica production in Canada and worldwide (Dixon and Tilston 2010; Peng et al. 2011; Strelkov et al. 2006). The resilient structure of the resting spores protects them from unfavorable environmental conditions and helps *P. brassicae* to survive in the soil for up to 17 years (Wallenhammar 1996). This longevity means that once a field becomes infested with *P. brassicae*, it is difficult to eradicate the pathogen. Moreover, the cultivation of canola in the same field year after year results in increases in inoculum levels. Many cultural practices, including sanitation, eradication of alternative hosts, the use of bait crops, crop rotation, avoiding compacted soils, application of lime, and soil solarization, have been evaluated for the management of clubroot (Ahmed et al. 2011; Donald and Porter 2014; Gossen et al. 2012; Hwang et al. 2014; McDonald et al. 2004; Murakami et al. 2002; Tremblay et al. 2005; Webster and Dixon 1991). The adoption of many of these practices, however, is constrained by either logistical or financial concerns (Gossen et al. 2013; Strelkov et al. 2012). Similarly, the use of synthetic pesticides, including fungicides and fumigants, although potentially effective, is prohibitively expensive, or associated with environmental and/or health concerns (Hwang et al. 2014; Liao et al. 2021). In this context, the deployment of CR canola cultivars has emerged as the most important and widely used clubroot management strategy (Peng et al. 2014; Rahman et al. 2014; Strelkov et al. 2016).

Clubroot-resistant canola cultivars were first introduced to Canada in 2009 and 2010, and until 2013 were highly effective against all pathotypes of *P. brassicae* reported from this country (Strelkov et al. 2016). However, novel strains of *P. brassicae* that could overcome the resistance

in CR canola cultivars were identified in two fields in central Alberta in 2013 (Strelkov et al. 2016). Since then, resistance-breaking strains of the pathogen have been found in hundreds of fields across Alberta and at least one field in Manitoba (Hollman et al. 2021; Strelkov et al. 2018; Strelkov et al. 2020). These strains include many new virulence phenotypes not detected before 2013, and the need to classify their reaction into pathotypes based on the host reactions led to the development of the Canadian Clubroot Differential (CCD) set (Strelkov et al. 2018). To date, 36 distinct pathotypes have been identified from Canadian collections of *P. brassicae*, 19 of which can overcome the resistance in most/all CR canola cultivars (Askarian et al. 2021; Hollman et al. 2021). Pathotype 3A is most common among these new pathotypes, followed by pathotype 3D (Hollman et al. 2021). Pathotype 3H, which was predominant before the introduction of the resistance trait, is also still commonly found (Hollman et al. 2021; Strelkov et al. 2018). Unlike pathotypes 3A and 3D, pathotype 3H is effectively controlled by the resistance in all CR canola cultivars on the Canadian market.

Given the emergence of new, highly virulent pathotypes of *P. brassicae*, the development of canola with durable resistance via the pyramiding of resistance genes could be important for clubroot management (Rahman et al. 2014; Yu et al. 2016)**.** The pyramiding of multiple *R*-genes in combination with genes with a minor effect (Ellis et al. 2014; Fukuoka et al. 2015) could increase the durability of resistance (Li 2016; Pilet-Nayel et al. 2017). Similarly, the rotation of *R*-genes may also be helpful in clubroot management, as this strategy has been effective for the control blackleg of canola/oilseed rape in France and Australia (Cornelsen et al. 2021). The identification and mapping of CR genes is a prerequisite for the pyramiding of more than one gene in elite commercial canola cultivars, as well as for the rotation of *R*-genes in canola

cropping systems. Most of the Brassicas serve as good sources of CR genes, except *B. juncea* and *B. carinata* (Deora et al. 2013).

Brassica rapa is an important source of clubroot resistance (Buczacki et al. 1975; Hatakeyama et al. 2017). In this species, several CR loci/genes have been mapped, including *Crr1, CRs, Rcr9, Rcr3, and Rcr9^{wa}* on chromosome A08, *Crr2* on A01, *Crr4* on A06 (Karim et al. 2020; Laila et al. 2019; Suwabe et al. 2003; Suwabe et al. 2006; Yu et al. 2017), *CRa, CRb, CRd, CRb^{kato}, Rcr1, RCr2, Rcr4, Rcr5, Crr3, CRk*, *PbBa3.1* and *PbBa3.3* on A03 (Chu et al. 2014; Hirai et al. 2004; Huang et al. 2019; Huang et al. 2017; Matsumoto et al. 1998; Pang et al. 2018; Piao et al. 2004; Saito et al. 2006; Sakamoto et al. 2008; Yu et al. 2016), and *CRc* and *Rcr8* on chromosome A02 (Sakamoto et al. 2008; Yu et al. 2017)

In addition, at least 22 quantitative trait loci (QTL) (*Pb-Bn1, Pb-Bn2, PbBn-01:60-1, PbBn-01:60-2, PbBn-01:60-3, PbBn-01:60-4, PbBn-01:07-1, PbBn-01:07-2, PbBn-01:07-3, PbBn-e4x04-1, PbBn-a-1, PbBn-l-1, PbBn-l-2, PbBn-k-1, PbBn-k-2, PbBn-k-3, PbBn-Korp-1, PbBn-Korp-2, PbBn-Korp-3, PbBn-Korp-4, and PbBn-Korp-5*) involved in clubroot resistance have been identified in *B. napus* (Hejna et al. 2019; Piao et al. 2009), along with the major CR locus *Pb-Bn1* (Manzanares-Dauleux et al. 2000). Nineteen QTL conferring resistance to seven isolates of *P. brassicae* were also detected on eight chromosomes of *B. napus* (Werner et al. 2008), among which four corresponded to the *B. rapa* genes *CRa, CRb, CRk* and *Crr3* and are located on chromosome A03 (Fredua-Agyeman & Rahman 2016; Piao et al. 2004; Piao et al. 2009; Zhang et al. 2016).

Major gene resistance has been identified only rarely in *B. oleracea.* Instead, resistance in this host is controlled by QTL (Laurens and Thomas 1993; Voorrips and Kanne 1997; Yoshikawa 1993), with a minor effect of two or more genes in the QTL (Landry et al. 1992;

Nomura et al. 2005; Pang et al. 2018; Rocherieux et al. 2004; Tomita et al. 2013; Voorrips and Kanne 1997). More than 37 QTL (*CR2a, CR2, pb-3, pb-4, QTL1, QTL3, QTL9, Pb-Bo1, Pb-Bo2, Pb-Bo3, Pb-Bo4, Pb-Bo5a, Pb-Bo5b, Pb-Bo8, Pb-Bo9a, Pb-Bo9b, PbBo1, Pb-Bo (Anju) 1, Pb-Bo (Anju)2, Pb-Bo (Anju)3, Pb-Bo (Anju)4, CRQTL-YC, CRQTL-GN_1, CRQTL-GN_2, DIC.I-1, DIC.II-1, Rcr7, PbC4.1, PbC6, PbC7.1, PbC7.2, PbC8, PbC9.1, PbC3, PbC4.2, PbC7.3, PbC9.2*) have been reported from *B. oleracea* (Dakouri et al. 2018; Farid et al. 2020; Lee et al. 2016; Nagaoka et al. 2010; Pang et al. 2018; Piao et al. 2009), which confer different degrees of clubroot resistance.

Molecular markers are widely used genetic tags to identify resistance genes as well as QTL. Initially, first-generation molecular markers, like restriction fragment length polymorphism (RFLP) (Landry et al. 1992), random amplified polymorphic DNA (RAPD) (Grandclément and Thomas 1996), amplified fragment length polymorphisms (AFLP) (Voorrips and Kanne 1997), and simple sequence repeats (SSR) (Kuginuki et al. 1999; Suwabe et al. 2006), were used to map CR genes. More recently, single nucleotide polymorphism (SNP) markers have been used in studies of genetic variation, genetic map construction, population structure analysis, association studies, map-based gene cloning, and other plant breeding applications (Rousseau-Gueutin et al. 2020; Dakouri et al. 2018; Song et al. 2020; Wang et al. 2020; Rajendran et al. 2022) . SNP markers are highly polymorphic and abundant throughout the genome, and SNP discovery with a reference genome is one of the most common high-throughput applications of next-generation sequencing (NGS) (Kumar et al. 2012).

Genotyping-by-sequencing (GBS) is one of the most promising NGS tools. It is used to provide rapid, inexpensive identification of SNP variants in many crops including Brassica species (Yu et al. 2017). It is also a very powerful tool for mapping a single gene responsible for

a specific trait, using high-density markers in the population of interest (Song et al. 2020; Wang et al. 2020; Rajendran et al. 2022). In GBS analyses, the extracted DNA is treated with restriction enzymes (REs). The REs cut the DNA into small pieces, which are ligated with barcode adapters and amplified. The amplified DNA fragments are sequenced through a high-throughput DNA sequencer and the data obtained are analyzed statistically and aligned to a reference genome to find variants (often SNPs). Illumina Hiseq and Ion torrent (Ion proton) sequencers are widely used NGS tools for GBS (Song et al. 2020; Mundada et al. 2022; Abed et al. 2022) and can be used to identify tens of thousands to hundreds of thousands of SNPs (Elshire et al. 2011). In addition to Illumina Hiseq 2000 and Ion torrent sequencing, other more powerful, long read (10,000 to 100,000 base pairs long) sequencing technologies (also called third-generation sequencing technologies), like Oxford Nanopore and PacBio Single-Molecule Real-Time (SMRT) sequencing, have been used to examine the genetic properties of many species with a complex genome structure. These long reads can span the repetitive region of a complex genome with a single continuous read (Istace et al. 2017), eliminating the complexities of the repetitive region (Rousseau-Gueutin et al. 2020) of the genome.

NGS technologies have been widely used to construct reference genomes for important crop species (Belanger et al. 2016;Rousseau-Gueutin et al. 2020; Song et al. 2020), including *Arabidopsis thaliana* (Schranz and Mitchell-Olds 2006), and almost all of the updated and published reference genomes for *B. rapa, B. oleracea, B. nigra, B. juncea* and *B. napus* (Bayer et al. 2017; Cai et al. 2017; Chalhoub et al. 2014; Liu et al. 2014; Parkin et al. 2014; Rousseau-Gueutin et al. 2020; Song et al. 2020; Song et al. 2021; Wang et al. 2011; Yang et al. 2016; Zhang et al. 2020). These reference genomes are crucial for identifying and mapping resistance genes (Delourme et al. 2018).

Genotyping-by-sequencing is highly specific, reproducible, and applicable to species with large, complex genomes (e.g., *Brassica* species with polyploid genomes), enabling the simultaneous identification and genotyping of SNPs (Song et al. 2020; Wang et al. 2020; Rajendran et al. 2022). The construction of high-resolution genetic maps (Mammadov et al. 2012) is also an important application of the technology, which could be used to identify linkage disequilibrium (LD) between loci and identify candidate genes through genome-wide association studies (GWAS) (Varshney et al. 2014).

GBS can help to detect SNPs by comparison with reference genomes, and so can help to identify and map clubroot resistance genes in *Brassica* species quickly and precisely. In clubroot research, GBS has been successfully used to identify and map many *CR* major genes and QTL (Laperche et al. 2017; Lee et al. 2016; Pang et al. 2018; Yu et al. 2016; Yu et al. 2017), helping to accelerate the development of CR cultivars through the enrichment of clubroot-resistant germplasm.

Canola (*Brassica napus* L.) is oilseed rape for human consumption and livestock feed, and contains with < 2% erucic acid in its oil profile and < 30 μmol glucosinolates per g of air-dried, oil-free meal. It is one of the most important edible oil crops worldwide, was originally derived from rapeseed (*B. napus*) through classical breeding. Erucic acid was eliminated from its oil profile, while glucosinolates were eliminated from the meal (the material remaining following extraction of the oil). Erucic acid was not of concern when rapeseed oil was used as an industrial lubricant, but is highly undesirable for human consumption because of its harmful effects on health (Bell 1982; Khachatourians et al. 2001). Similarly, glucosinolates in the meal, which is used as livestock feed, are harmful to animal health (Bell 1982; Khachatourians et al. 2001).

Today, canola is widely grown in Canada and across the world for its high-quality edible oil and low glucosinolate content meal.

This chapter reviews the development of canola, its importance to the Canadian economy, challenges in canola production, the application of NGS technologies to overcome these challenges, and the identification and mapping of disease resistance genes with an emphasis on clubroot.

1.2. Literature Review

1.2.1. The *Brassica* genus and Triangle of U

Brassica is a genus in the family Brassicaceae (previously known as the Cruciferae) (Cheng et al. 2014; Warwick et al. 2006). *Brassica* species are among the oldest cultivated plants and can be traced back to 5000 BCE in the archaeological record and 1500 BCE in the written record (Raymer 2002). Historically, crops in this genus were grown as oilseeds, forages, condiments, and vegetables. Three amphidiploid *Brassica* species evolved from the hybridization of diploid progenitors (U 1935) through natural hybridization and a subsequent doubling of diploid chromosomes. The Triangle of U (Figure 1.1.), first proposed in the early 20th century (Morinaga 1934; U 1935; cited by Raymer et al. 2002), illustrated the relationship of *B. juncea*, *B. carinata* and *B. napus* with their diploid progenitors, *B. nigra*, *B. rapa* and *B. oleracea*. In this model, the amphidiploid species *B. juncea* (genome characterized as AABB, 2*n* = 36) resulted from the interspecific hybridization of two diploid species *B. nigra* (BB, 2*n* = 16) and *B. rapa* (AA, 2*n* = 20); *B. carinata* (BBCC, 2*n* = 34) resulted from the hybridization of *B. nigra* (BB, 2*n* = 16) and *B. oleracea* (CC, 2*n* = 18); and *B. napus* (AACC, 2*n*=38) resulted from the hybridization of *B. rapa* (AA, 2*n* = 20) and *B. oleracea* (CC, 2*n* = 18) (U 1935; Raymer 2002).

Among the amphidiploid species, *B. napus* is considered the most ancient, followed by *B. juncea* and *B. carinata* (U 1935).

1.2.2. Major Brassica crops

1.2.2.1. *Brassica rapa* (AA, 2*n*=20)

The diploid species *B. rapa* originated in the Mediterranean region and gradually spread across Scandinavia, Germany, Central Europe, and eventually to Asia (Mizushima and Tsunoda 1967; cited by Dixon 2007), especially Japan, China, and India. It is believed to be the first domesticated species of *Brassica* (Gómez-Campo and Prakash 1999). Different varieties or subspecies of *B. rapa* have been widely grown as vegetables and for oil production. Genetic diversity in *B. rapa* is high, and efforts to broaden the genetic diversity in *B. napus* have included interspecific hybridization with and the exploitation of the genetic variability in *B. rapa* (Qian et al. 2006; Annisa et al. 2013)). In Canada, this species has been cultivated since the first half of the 20th century, when an immigrant farmer (Mr. Fred Solvoniuk) brought seeds of *B. rapa* from his native Poland to Saskatchewan (Bell 1982-). Many of the original canola-quality (low erucic acid and low glucosinolates) Brassica genotypes in Canada were *B. rapa*, and were often referred to as 'Polish canola' given the Polish origin of the original seed.

1.2.2.2. *Brassica oleracea* (CC, 2*n* = 18)

Brassica oleracea includes several economically important Brassica vegetables, such as broccoli, Brussels sprouts, cabbage, cauliflower, collards, kale, and kohlrabi. These vegetables are a good source of vitamins, proteins and carotenoids for human consumption. Kale and cabbage originated as early as 2000 BC and are believed to be the first cultivated crops of this species in Europe (Chiang et al. 1993), from which many leafy vegetables and heading cabbages have probably descended. The species was eventually introduced to Asia, where it gave rise to

Chinese kale (*B. oleracea* var. *alboglabra*) (Hervé 2003; Maggioni 2015). Cauliflower and broccoli are thought to have originated in the Mediterranean region (Branca 2008). The *B. oleracea* genome has experienced several rounds of whole-genome duplication followed by frequent gene shuffling and the loss of many genes. The genome is an estimated 630 Mb in size, with 45,758 protein-coding genes identified in an assembly providing 85% coverage of the whole genome (Guo et al. 2021). These included 5,107 retrotransposons and 8,527 DNA transposons that can produce variants through alternate splicing and intron skipping (Liu et al. 2014; Guo et al. 2021).

1.2.2.3. *Brassica nigra* (BB, 2*n* = 16)

Brassica nigra is one of the progenitors of the amphidiploid species *B. carinata* and *B. juncea*. It is grown mainly as condiment mustard (black mustard), although it has largely been replaced for this purpose by *B. juncea*, because *B. juncea* produces higher yields than *B. nigra* of similar quality. It is believed that *B. nigra* originated in Europe and spread to Asia, especially India. In Canada, the species has largely been grown as an annual crop across Canada, from British Columbia to Newfoundland (Brouillet et al. 2010).

The 'B' genome of *B. nigra* is an important resource for many agronomic qualities, fatty acid composition, and glucosinolate content in the seed meal (Chèvre et al. 1991; Struss et al. 1991b), disease resistance (Zhu et al. 1993), and drought and heat tolerance. Therefore, researchers have introgressed useful genes from *B. carinata* or *B. juncea*, particularly related to disease resistance, by crossing with other cultivated Brassicas (Mei et al. 2022). Since the compatibility of the B-genome with the two other amphidiploid species is generally not a major issue, *B. nigra* is a valuable source of resistance traits.

1.2.2.4. *Brassica juncea* (AABB, 2*n* = 36)

Brassica juncea is an amphidiploid species that arose by the interspecific hybridization of *B. rapa* (AA) and *B. nigra* (BB), followed by a spontaneous doubling of the chromosomes. The species has been cultivated in China from 4000 to 5000 BC and in India since 2300 BC (Yang et al. 2016). Many sub-varieties have been developed in this species to improve its vegetable and edible oil characteristics. In many parts of China, *B. juncea* is grown as a vegetable crop (Zhang et al. 2015). In India, Bangladesh, Pakistan, Nepal, western Egypt, central Asia, and southern and southeastern Russia, *B. juncea* has been mainly cultivated as a seed or oil crop (Kumar et al. 2012; Wijesundera et al. 2008). In Canada, *B. juncea* has been cultivated as a mustard crop for many years because of its drought tolerance. Some canola-quality *B. juncea* cultivars also have been developed because of their tolerance to heat and drought, reduced pod shattering, and disease resistance (Burton et al. 2007; Potts et al. 2003). The crop is a complimentary oilseed crop to *B. napus* (canola) for cultivation in hot and low-rainfall areas, where canola production is difficult. The *B. juncea* genome has been sequenced and has an estimated size of 954.90 Mb (Yang et al. 2016).

1.2.2.5. *Brassica carinata* (BBCC, 2*n* = 34)

The cultivation of *B. carinata* began in Ethiopia around 4000 BCE and gradually spread to southern Europe and India (Prakash et al. 2012). Today, commercial cultivation is limited mainly to Ethiopia and the surrounding countries (Marillia et al. 2014; Seepaul et al. 2021). Nonetheless, *B. carinata* has been grown in Montana, North Dakota, and in southern parts of the United States such as Mississippi and Florida (Marillia et al. 2014). The crop was first cultivated in Canada in the mid-1980s, as a potential alternative to other oilseed crops (Getinet et al. 1997; Rakow and Getinet 1997). *B. carinata* is mostly self-pollinated, but cross-pollination at nearly 30% has been

reported in some cases (Cheung et al. 2015), likely associated with delayed anthesis. The seeds have about 48% oil content with a high erucic acid fraction in the oil profile, making *B. carinata* a good source of biofuels, plastics, and lubricants for industrial purposes (Velasco et al. 2003). The B-genome of *B. carinata* is a good source of resistance genes against blackleg (*Leptosphaeria maculans*) (Rimmer and Van den Berg 1992) and white rust (*Albugo candida*) (Kole et al. 2002), but it is most susceptible to Alternaria leaf spot (*Alternaria brassicae)* (Sharma et al. 2002) and clubroot (Peng et al. 2013).

Brassica carinata can serve as a bridging species to transfer a gene from the wider *Brassica* gene pool to a specific species. For example, a hexaploid hybrid (AABBCC) has been derived from an interspecific cross between *B. carinata* (BBCC, 2*n* = 34) and *B. rapa* (AA, 2*n* = 20), which could be used as a bridge hybrid for gene transfer as well as to develop a hybrid variety (Li et al. 2004; Abdeta 2022).

1.2.2.6. *Brassica napus* (AACC, 2*n*=38)

As noted earlier, *B. napus* (canola or rapeseed) is one of the most important oilseeds worldwide, providing 13% to 16% of the global demand for vegetable oil (Song et al. 2020). Most commercial canola cultivars in Canada are *B. napus*. Cultivated *B. napus* is believed to have arisen within the past 10,000 years (Wang et al. 2011) in the Mediterranean region (Raymer 2002) through several rounds of natural interspecific hybridization between *B. rapa* and *B. oleracea*. *B. napus* is a self-pollinating (12% to 47% cross-pollination can occur under favorable conditions) (Becker et al. 1992), amphidiploid (AACC; 2*n* = 38) species (Gómez-Campo 1999).

The 19 chromosomes of *B. napus*, comprising a genome of about 1.13 Gb, are descended from the progenitor species *B. rapa* (AA, 2*n*=20) and *B. oleracea* (CC, 2*n*=18). Genome sequencing indicated that the C genome descended from *B. oleracea* was larger than the A

genome from *B. rapa*, and that about a third of the genome is comprised of transposable elements (TEs) (Chalhoub et al. 2014). Several *B. napus* reference genomes obtained by short-read (Chalhoub et al. 2014) and long-read sequencing (Rousseau-Gueutin et al. 2020; Song et al. 2020), as well as pangenomes (Bayer et al. 2020), have been published.

1.2.3. Canola and its development in Canada

Lines of *B. napus* with high erucic acid and high glucosinolate content have been grown in Canada since 1936. During the Second World War, production increased sharply due to its high demand as a lubricant for industrial use, and research on quality improvement also increased to make the oil and meal suitable for consumption as food and feed (Khachatourians et al. 2001). The first breakthrough came in 1963, when Professor Baldur Stefansson of the University of Manitoba, Canada, identified *B. rapa* line 'Liho' with low erucic acid (Downey 1990).

Canola ('Canadian oil low acid') is oilseed rape for human consumption and livestock feed with oil that contains < 2% erucic acid and meal that contains < 30 μmol glucosinolates per g of air-dried, oil-free meal (Canola Council of Canada. 2014). The first low erucic acid cultivar of *B. napus*, 'ORO', was released in Canada in 1968 (Bell 1982; Khachatourians et al. 2001). However, 'ORO' still contained a significant amount of glucosinolates in its meal. The *B. napus* cv. 'Bronowski' was identified from Poland (Bell 1982; Khachatourians et al. 2001) and found to have a low glucosinolates content. 'Bronowski' was used in a backcross program to develop a low glucosinolate and low erucic acid canola cultivar. In 1974, the first low erucic acid and low glucosinolates canola cultivar 'Tower' (*B. napus*) was released in Canada by Dr. Stefansson of the University of Manitoba, Winnipeg, MB (Bell 1982), followed by the canola-quality *B. rapa*

cultivar ‘Candle’ in 1977 by Dr. R. K. Downey of Agriculture and Agri-Food Canada, Saskatoon, SK (Bell 1982).

The development of ‘Tower’ and ‘Candle’ opened the way for the commercial cultivation of canola in Canada, with the name ‘Canola’ registered as a trademark to refer to “Seeds of the genus *Brassica* (*B. napus, B. rapa or B. juncea*) from which the oil shall contain less than 2% erucic acid in its fatty acid profile and the solid component shall contain less than 30 μmol of any one or any mixture of 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3 butenyl glucosinolate, and 2-hydroxy- 4-pentenyl glucosinolate per gram of air-dry, oil-free solid” (Canola Council of Canada 2014a). The United States Food and Drug Administration (FDA) granted canola oil Generally Recognized as Safe (GRAS) status for human consumption in 1985 (Brown 2008).

1.2.4. Economic importance of canola in Canada

Canada is the largest producer of canola worldwide, with about 18.6 million metric tonnes of grain produced per year (Canola Council of Canada, 2019). Since the development of canola in the 1970s, the cultivation of canola in Canada has increased sharply with some minor fluctuations (Figures 1.2 & 1.3). The crop contributes about $26.7 billion CAD to the national economy (Canola Council of Canada 2020). The crop is grown mainly on the Prairies, where Saskatchewan contributes 54% of production, Alberta 29%, and Manitoba 16% (Canola Council of Canada, 2019 /). Prior to 2019, China was the number one importer of canola seed and oil from Canada; but in 2019, the United States become the major importer of Canadian canola (Canola Council of Canada 2020-).

1.2.5. Major challenges in canola production

Canola is an economically important and nutritionally rich crop, in Canada and worldwide (Dixon 2014). Winter-type canola is grown mainly in Europe and semi-winter type in southern China, while the spring-type is grown in Canada, Australia, the USA, northern Europe, and northern China (Friedt et al. 2007). Many factors influence canola production, including the cultivars, agronomic practices, environmental conditions, diseases and pests, and vernalization requirements (for winter types) (Canola Council of Canada 2020). Among these factors, clubroot disease has emerged as one of the most important issues for canola in Canada. Clubroot, caused by the obligate parasite *Plasmodiophora brassicae* Wor., causes significant yield losses, estimated at 15% to 30% in susceptible crops, but occasionally resulting in total (100%) loss (Strelkov et al. 2007; Tewari et al. 2005).

1.2.6. Clubroot and its causal agent

1.2.6.1 Clubroot disease

Clubroot is believed to have originated in the Mediterranean region, together with many of its Brassica hosts (Howard et al. 2010). The disease has been known for many centuries. Symptoms resembling clubroot were described on cabbage in Spain in 1539, and the disease was first reported in England in 1736, where it was found to cause yield loss in turnip (Watson and Baker 1969). By the late 1860s, clubroot was causing significant yield losses near St. Petersburg, Russia, and in 1878, the Russian botanist Mikhail Woronin first described the detailed characteristics of the disease and its causal agent, *P. brassicae* (Woronin 1878). Clubroot now occurs in most Brassica-growing regions worldwide, including the Americas, Australia, Europe, and Asia (Dixon 2009). The disease was likely introduced to Canada by European settlers

(Sedaghatkish et al. 2019) and was primarily reported on cabbage, cauliflower, and turnip crops in Ontario, British Columbia, Quebec, and Atlantic provinces (Conners 1945) for many decades.

Despite the widespread cultivation of canola since the 1970s, clubroot was not identified in this crop on the Prairies until 2003, when a dozen infested fields were found near Edmonton, Alberta (Tewari et al. 2005). The disease has continued to spread over the past 18 years, with over 3000 confirmed field infestations in Alberta, and increasing numbers of cases in Saskatchewan, Manitoba, and North Dakota (Cao et al. 2009; Chapara et al. 2019; Chittem et al. 2014; Hollman et al. 2021; Strelkov et al. 2020).

Initially, management strategies were limited mainly to sanitization of field equipment and long rotations out of canola (Strelkov and Hwang 2014). In 2009–2010, however, the first clubroot-resistant (CR) commercial canola cultivars were released. These cultivars provided excellent protection against the predominant pathotypes of *P. brassicae*, and soon became the most important clubroot management tool. Unfortunately, new pathotypes of *P. brassicae* able to overcome this resistance quickly emerged and proliferated (Strelkov et al. 2016; Strelkov et al. 2018), and now represent a challenge for sustainable management of the crop (Hollman et al. 2021; Strelkov et al. 2020).

1.2.6.2. Taxonomy and pathotypes of *Plasmodiophora brassicae*

The clubroot pathogen can grow and reproduce only on living hosts (see below) and cannot (to date) be cultured on an axenic medium. It is classified in the Rhizaria supergroup of the Eukaryota (Burki et al. 2010), and shares numerous characteristics with other Plasmodiophorids like *Spongospora subterranea* (powdery scab of potato) and *Polymyxa graminis* (parasite of various cereals and a vector of the Barley yellow mosaic virus). *Plasmodiophora brassicae* exhibits cruciform nuclear division, produces zoospores with two

anterior whiplash-like flagella, and forms multinucleate plasmodia and resting spores (cysts) with chitin in the cell walls (Braselton 1995; Dylewski and Miller 1983; Ingram and Tommerup 1972; Kageyama and Asano 2009).

Physiologic specialization occurs in *P. brassicae*, meaning that the parasite exists as multiple races or pathotypes that differ in their capacity to infect specific host genotypes (Karling 1968). Isolates of *P. brassicae* are classified into pathotypes based on their virulence patterns on defined groups of hosts, referred to as differential sets. Numerous differential sets have been developed over the past 60 years to identify *P. brassicae* pathotypes. The most widely used differentials include those of (Williams 1966), which consist of two rutabagas and two cabbage cultivars, the differentials of (Somé et al. 1996), originally developed to identify pathotypes recovered from oilseed rape in France, and the European Clubroot Differential (ECD) set (Buczacki et al. 1975), which was an attempt to develop an international classification system. In Canada, all three systems have been used at different times to characterize *P. brassicae* isolates recovered from canola and vegetable Brassicas (reviewed in Strelkov and Hwang 2014).

Recently, the Canadian Clubroot Differential (CCD) set (Strelkov et al. 2018) was developed for Canada in response to the identification of isolates that could overcome the resistance in CR cultivars, but which could not be distinguished from existing pathotypes based on their virulence on the existing differential systems (Strelkov et al. 2016). The CCD set consists of 13 host genotypes, including the differentials of Williams and Somé et al., selected hosts of the ECD set, and several additional *B. napus* canola/rapeseed hosts of particular interest to canola production in Canada. The inclusion of the differentials of Williams and Somé et al. allows pathotype designations to be obtained as per those systems, providing continuity with existing literature and earlier studies. Originally, *P. brassicae* isolates were assigned a single

uppercase letter to designate their CCD classification (Strelkov et al. 2018). Given the rapid identification of many new pathotypes, however, the pathotype nomenclature was revised slightly to include both a number denoting the Williams' designation, followed by a letter indicating the CCD classification (Askarian et al. 2021). The entire alphabet may be applied to distinguish different variants of a single Williams' pathotype (e.g., pathotype 2A, 2B, 2C), thereby accommodating a very large number of potential pathotypes. To date, 36 pathotypes of *P. brassicae* have been identified from Canada (Askarian et al. 2021; Hollman et al. 2021; Strelkov et al. 2018; Strelkov et al. 2020), 19 of which can overcome the resistance in at least some CR canola cultivars.

1.2.6.3. Lifecycle of *Plasmodiophora brassicae*

Resting spores of *P. brassicae* can survive in the soil for many years in the absence of a host (Dixon 2009; Wallenhammar 1996). However, more recent work indicates that up to 90% of the resting spores may disappear in the two years following a canola crop, with a small proportion of the spore population surviving much longer (Ernst et al. 2019; Gossen et al. 2019; Peng et al. 2015). The pathogen life cycle consists of three distinct stages: 1) survival and germination of the resting spores in the soil, 2) infection of the host root hairs, and 3) infection of the root cortex (Ingram and Tommerup 1972). Following resting spore germination, primary zoospores are released to infect the root hairs. Once in the root hairs, the pathogen forms primary plasmodia that give rise to secondary zoospores. These zoospores infect the root cortical tissue, forming intracellular secondary plasmodia. This stage is associated with the formation of the root galls and visible symptoms. Eventually, the plasmodia are cleaved into new resting spores, which are released into the soil when the galls decompose (Braselton 1995; Ingram and

Tommerup 1972; Kageyama and Asano 2009). The lifecycle of *P. brassicae* is summarized in Figure 1.4.

Soil moisture, temperature, the depth of the spores in the soil profile, host susceptibility, and soil pH influence the longevity and germination rate of the resting spores. Among these various factors, soil pH plays a particularly important role. The rate of spore germination increases in acidic soil compared with alkaline soil. The most suitable pH for zoospore development is between 5.4 and 7.1 (Myers and Campbell 1985; cited by Donald et al. 2009). The resting spore density also depends on the depth of the soil. Most (> 97%) resting spores are found in the upper layer (0-5 cm depth) of the soil, with fewer spores found below a depth of 40 cm (Cranmer et al. 2017; Dixon 2009; Kim et al. 2000). Temperature, soil moisture, and the presence or absence of plant root exudates also greatly influence resting spore germination and movement of zoospores. The resting spore germination rate is highest at 24°C at a pH of 6.0–6.7 (Myers and Campbell 1985; cited by Donald et al. 2009). Soil moisture also plays a significant role, facilitating the passive movement of the zoospores without the need to spend much energy to reach the root hairs (Horiuch & Hori 1980; Kageyama and Asano 2009). This might be a reason why zoospores need sufficient soil moisture to colonize the root hairs successfully. Resting spore germination is also enhanced by the presence of root exudates released by host (and some non-host) plants (Dixon 2009; Friberg et al. 2005; Rashid et al. 2013).

A primary zoospore is spindle-shaped or pyriform, biflagellate, about 2.8 µm to 5.9 µm long, and sometimes spiny in structure (Kageyama and Asano 2009). One flagellum of the biflagellate zoospores is short with a blunt end and the other one is longer with a whiplash structure or tailpiece. After germination of the resting spores, the zoospores reach the root and attach to the root hairs (Kageyama and Asano 2009). Each zoospore, with all of its contents,

flattens and the flagella coil around the flattened zoospore. The zoospore then develops a sharp structure, along with a tubular vesicle, called the 'rohr'. The 'rohr' is a sharp-tipped structure in which one end is bounded by a plasma membrane and the sharp end, called the 'stachel', is pointed towards the host cell to inject the spore material into the root cell. The process of injecting pathogen protoplasm into the host cell relies primarily on the physical force created by the spore; no enzymatic activity is required to complete infection (Ingram and Tommerup 1972; Kageyama and Asano 2009; Laila et al. 2020).

Following infection by the resting spores, the second stage of the life cycle is the colonization in the root hairs. In each root hair, the pathogen forms a primary plasmodium (a naked mass of protoplasm with multiple nuclei in the single-cell of an obligate parasite). Several nuclear divisions occur synchronously in the plasmodium to produce the multinucleate primary plasmodium over several days, followed by its cleavage into zoosporangia. Each sporangium contains 14-16 secondary zoospores, which are released back into the soil to infect the root cortex (Howard et al. 2010; Ingram and Tommerup 1972). The secondary zoospores cannot be visually differentiated from the primary zoospores and, to date, no differences in the structure of primary and secondary zoospores have been described (Ingram and Tommerup 1972; McDonald et al. 2014).

Following penetration of the cortical tissues, *P. brassicae* initiates the formation of intracellular secondary plasmodia. Physiological and morphological changes associated with this secondary stage of infection result in the development of the galls or clubs typical of clubroot disease (Garber and Aist 1979; Kageyama and Asano 2009). In the root cortex, the pathogen causes hormonal imbalances (particularly cytokinin and auxin) that play a significant role in the progress of the disease and symptom expression (Figure 1.4) (Laila et al. 2020). The

changing auxin and cytokinin concentrations stimulate host cell division, cell expansion, and loss of host tissue differentiation, ultimately leading to the development of root galls. The root galls become nutrient sinks, reducing the grain fill and quality, while at the same time also reducing root functionality and restricting water and nutrient uptake by the plant. Finally, the multinucleate plasmodia undergo a cleavage process to develop into resting spores (Ikegami 1992; cited by Kageyama and Asano 2009), which are released back into the soil after the decomposition of the infected roots. These resting spores serve as the inoculum for future infections (Kageyama and Asano 2009; Williams and McNabola 1967).

1.2.7. The emergence of clubroot in Canadian canola

The history of clubroot in Canada is obscure, but *P. brasssicae* was likely introduced in infected fodder turnips brought by European settlers (Howard et al. 2010). The presence of the disease on vegetable Brassicas was documented by the early 20th century in British Columbia (Rankin & Fraser 1920; cited by Howard et al. 2010), Quebec, the Maritimes, and Ontario (Conners 1945; cited by Howard et al. 2010). In 1997, clubroot was detected for the first time on canola in the Lac St. Jean region of Quebec (Howard et al. 2010). However, while there were sporadic reports of the disease in home and market gardens on the Prairies going back decades (Howard et al. 2010), clubroot was not observed on canola in western Canada until 2003 (Tewari et al. 2005). That year, the disease was identified in a dozen fields near Edmonton, Alberta. Since then, it has continued to spread across the province, with more than 3300 confirmed field infestations by 2019 (Strelkov et al. 2020). It is now also found with increasing frequency in Saskatchewan, Manitoba, and North Dakota (Chapara et al. 2019; Chittem et al. 2014), as well as in Ontario (Al-Daoud et al. 2018), indicating that clubroot is becoming an issue across much of Canada (McDonald et al. 2021) and the Northern Great Plains.

When clubroot was first identified on canola in western Canada, few management options were available to growers. These were restricted mainly to long rotations out of susceptible hosts, and the sanitization of field equipment to slow down the spread of the pathogen (Strelkov et al. 2012). Other management methods were explored, including the application of soil fumigants, deployment of bait crops, and the use of soil amendments such as wood ash and lime (Hwang et al. 2014). However, while these strategies may have had some success in the reduction of clubroot on cruciferous vegetables, they were not practical or economical in canola cropping systems (Hwang et al. 2014). As such, the use of genetic resistance to clubroot in Canadian canola cultivars has become the cornerstone of clubroot management in canola in Canada.

The first CR canola cultivar became available in 2009, followed by many other cultivars in 2010 and subsequent years. All of these cultivars were highly resistant to the predominant pathotypes of *P. brassicae* found in Canada, including pathotype 3 as defined on the system of Williams (1966), which was most common on canola before the introduction of the resistance trait (Strelkov et al. 2007; Strelkov et al. 2006; Xue et al. 2008). The CR canola cultivars soon became the main (and in many cases the only) clubroot management tool used by farmers (Peng et al. 2014; Rahman et al. 2014). Unfortunately, the cultivation of resistant hosts in short-rotation can exert strong selection pressure on *P. brassicae* populations, resulting in rapid shifts in virulence and a loss in the effectiveness of resistance (LeBoldus et al. 2012; Peng et al. 2014). In 2013, novel strains of *P. brassicae* that could overcome the resistance in most CR canola cultivars were identified from two fields in central Alberta (Strelkov et al. 2016). Over the past 8 years, resistance-breaking pathotypes of *P. brassicae* have been recovered with increasing frequency from hundreds of fields across Alberta, and in at least one field in Manitoba (Hollman

et al. 2021; Strelkov et al. 2018; Strelkov et al. 2020). As noted earlier, 36 pathotypes have been identified in Canada, of which 19 can overcome the resistance in at least some CR canola hosts. These include pathotypes 3A and 3D, which are among the most prevalent, as well as pathotype 5X, which was the first of the resistance-breaking pathotypes to be identified (Hollman et al. 2021; Strelkov et al. 2018). The original pathotype 3, now classified as pathotype 3H, is also still commonly found, particularly in regions where CR canola has not been extensively deployed (Hollman et al. 2021). It is clear that with the rapid emergence of new pathotypes, there must be an effort to identify novel sources of resistance and to improve understanding of the genetics of the host-pathogen interaction.

1.2.8. Resistance to clubroot

1.2.8.1 General model of resistance

Resistance is the inherent capacity of a host to prevent, suppress, or slow-down disease development (Arya and Sharma 2016). Resistance in plants includes several layers of defense. In the first line of defense, the plant recognizes a pathogen through various receptors, known as pattern recognition receptors (PRRs), which interact with conserved microbe-associated molecular patterns (MAMPs) (Jones and Dangl 2006). When recognition occurs, a general defense response is initiated, which can include mechanisms such as cell wall alterations and the accumulation of defense-related compounds. Some pathogens can overcome this generalized resistance response by producing molecules (effectors) that suppress host defenses. This is known as effector-triggered susceptibility (ETS). Plants, in turn, have evolved other receptors, which are often R-proteins encoded by *R*-genes, that recognize these effectors and trigger effector-triggered immunity (ETI). This consists of a very strong resistance response that

prevents infection. Pathogens may alter or delete the recognized effectors to avoid eliciting ETI, but this could have a fitness cost to the pathogen in the absence of the host *R*-genes.

1.2.8.2 Genetic resistance to clubroot

The primary zoospores of *P. brassicae* can infect root hairs of both susceptible and resistant hosts, as well as many non-host (i.e., non-crucifer) species (Deora et al. 2013; Feng et al. 2012; Ludwig-Müller et al. 1999). It is during secondary infection of the cortex that differences in the reaction of resistant and susceptible hosts become evident. There is no or little development of the pathogen in the cortex of resistant cultivars (Deora et al. 2013; Gossen et al. 2013; Kroll et al. 1983).

A hypersensitive response was reported as an important disease resistance mechanism in *B. rapa* (turnip) and *Arabidopsis* (Deora et al. 2012), but was not observed in Chinese cabbage, cauliflower (Donald et al. 2008; Kroll et al. 1983; Tanaka et al. 2006) or canola (Gossen et al. 2013). Nonetheless, the production of reactive oxygen species (ROS) near the endodermis creates an extra barrier (Deora et al. 2012) to colonization by the pathogen and disease progression in resistant hosts. The ROS activate oxidative burst-related enzymes and signaling molecules that modulate transcription factors to cause cell death and activate defense responses in the *P. brassicae*-Brassica interaction (Agarwal et al. 2011; Stael et al. 2015; Zhao et al. 2017). Restriction of gall enlargement, associated with the development of spheroid galls (Buczacki et al. 1975; Osaki et al. 2008; Rennie et al. 2013; Williams 1966;), represents another resistance mechanism in host plants. While *P. brassicae* development is severely constrained in the spheroid galls, the pathogen can proliferate and infected tissues can expand in the more typical spindle-shaped galls (Rennie et al. 2013). Since starch plays a vital role in providing energy (carbon) to the pathogen (Donald et al. 2008; Keen and Williams 1969), resistant plants may also

reduce starch production to restrict the growth of *P. brassicae* and slow clubroot progression (Tanaka et al. 2006).

Resistant plants that carry *R*-genes often upregulate jasmonate and ethylene metabolism, increase callose deposition in the cell walls, and produce indole-containing metabolites as defense mechanisms against *P. brassicae* (Galindo-González et al. 2020). The upregulation of a salicylic acid (SA)-mediated response, together with defense-related proteins such as chitinases and thaumatins, has also been reported as part of the resistance reaction of host plants to *P. brassicae* (Galindo-González et al. 2020; Zhou et al. 2020).

Resistance to clubroot can be qualitative (controlled by major (*R*) genes) (Crute et al. 1980) or quantitative (controlled by quantitative trait loci (QTL)) (Figdore et al. 1993; Voorrips and Kanne 1997). Qualitative resistance controlled by *R*-genes provides strong resistance to specific pathotypes of *P. brassicae*, while quantitative resistance provides some level of resistance to a wider range of pathotypes. At present, almost all of the CR canola cultivars in Canada appear to carry pathotype-specific resistance (*R*-gene) controlled by a single dominant gene (Peng et al. 2014; Rahman et al. 2014). The source of this resistance is believed to be derived from the European winter *B. napus* 'Mendel' (Fredua-Agyeman et al. 2018). The monogenic CR cultivars developed and released in 2009, with a dominant resistant gene from the winter cultivar 'Mendel', showed strong resistance to almost all of the known pathotypes before 2013. The identification of a growing number of pathotypes able to overcome resistance, beginning in 2013 and continuing to the present, likely reflects the selection pressure imposed on *P. brassicae* populations by the monogenic resistant cultivars (Fredua-Agyeman et al. 2018; Strelkov et al. 2016). Given the widespread cultivation of CR canola cultivars, 'Mendel'-derived

resistance has been overcome in an increasing number of fields in Canada (LeBoldus et al. 2012; Peng et al. 2014; Strelkov et al. 2016).

1.2.9. Identification and mapping of clubroot resistance genes

Resistance genes function primarily against specific pathotypes of *P. brassicae* (Ayers and Lelacheur 1972; Diederichsen et al. 2003; Piao et al. 2004; Voorrips and Visser 1993), but QTL have also been identified (Diederichsen et al. 2009; Hirai 2006; Voorrips and Kanne 1997) that confer resistance to the pathogen. While almost all of the *Brassica* species have been studied to identify resistance genes effective against clubroot, most studies have focused on the three most economically important Brassicas: *B. rapa*, *B. oleracea*, and *B. napus*. The A genome of *B. rapa* is the primary source of *R*-genes (Crute et al. 1980; James and Williams 1980; Wit and Van de Weg 1964). In contrast, *B. oleracea* (C genome) primarily carries QTL for resistance, while *B. napus* carries both major genes and QTL on the A and C genomes (Figdore et al. 1993; Grandclément and Thomas 1996; Voorrips 1996). Clubroot resistance gene identification has been extended to the B genome of *B. nigra*, with the resistance QTL *Rcr6* identified from *B. nigra* homologous to the position of chromosome A08 in *B. rapa* (Chang et al. 2019). There is limited information regarding clubroot resistance in *B. juncea* or *B. carinata* (Diederichsen et al. 2009; Fredua-Agyeman et al. 2019).

1.2.9.1 Clubroot resistance in *B. rapa*

Early studies of European fodder turnip demonstrated that it is a strong source of clubroot resistance genes (Buczacki et al. 1975; Crute et al. 1983; Karling 1968; Matsumoto et al. 1998). Similarly, *B. rapa* genotypes were selected for inclusion in the ECD set (ECD 01, ECD 02, ECD 03, and ECD 04 in particular) because they carried important sources of resistance (Buczacki et al. 1975; Toxopeus and Janssen 1975; Diederichsen et al. 2003; Hirani et al. 2018; Fredua-

Agyeman et al. 2020; Yu et al. 2021) mediated by *R*-genes (Ueno et al. 2012). Since then, many additional CR loci/genes (*CRa, CRb*, *Crr3*, *Rcr1, Rcr2, Rcr9, Rcr9*^{wa,} and others) have been identified in *B. rapa*. These CR loci/genes mainly encode the NBS-LRR family of genes (Chu et al. 2014; Hatakeyama et al. 2013, 2017; Ueno et al. 2012), although not all of the genes have been cloned; the NBS-LRR proteins encoded by these genes may recognize pathogen effectors to activate the plant immune system (Bernoux et al. 2011).

The introgression of a clubroot resistance gene from turnip to Chinese cabbage indicated that the turnip cultivars 'Gelria', 'Mommersteeg', and 'Waaslander' carried at least three dominant, pathotype-specific genes for resistance to *P. brassicae* (Toxopeus and Janssen 1975; Wit and Van de Weg 1964). Another European turnip cultivar 'Siloga' carried the *Crr1, Crr2,* and *Crr4* QTL/genes for resistance to specific isolates of *P. brassicae* (Hatakeyama et al. 2013; Suwabe et al. 2003; Suwabe et al. 2006). Based on these studies, it is clear that clubroot resistance in *B. rapa* is controlled by one gene or more than one gene.

More recent publications have identified and mapped 18 resistance loci on the A genome of *B. rapa* (Diederichsen et al. 2009; Hatakeyama et al. 2017; Karim et al. 2020; Piao et al. 2009; Sakamoto et al. 2008; Yu et al. 2017), which provide pathotype-specific resistance to *P. brassicae*. Most of these loci have been identified on chromosome A03, followed by A08, A02, A01, and A06. The resistance genes/loci on chromosome A03 include *CRa* (Matsumoto et al. 1998), *CRb* (Piao et al. 2004), *CRd* (Pang et al. 2018) *CRk* (Sakamoto et al. 2008), *Crr3* (Hirai et al. 2004; Saito et al. 2006), *PbBa3.1* and *PbBa3.3* (Chen et al. 2013), *Rcr1* (Chu et al. 2014), *Rcr2* (Huang et al. 2017), *Rcr4* (Yu et al. 2017), and *Rcr5* (Huang et al. 2019). On chromosome A08, the resistance gene/loci include *Crr1* (Suwabe et al. 2003), *CRs* (Laila et al. 2019), *Rcr9* (Yu et al. 2017), *Rcr3* (Karim et al. 2020), and *Rcr9*^{wa} (Karim et al. 2020). Resistance loci on

chromosome A02 include *CRc* (Sakamoto et al. 2008) and *Rcr8* (Yu et al. 2017), while on chromosomes A01 and A06, they include *Crr2* (Suwabe et al. 2006) and *Crr4*, respectively (Suwabe et al. 2006).

1.2.9.2. Clubroot resistance in *Brassica oleracea*

Unlike *B. rapa*, clubroot resistance in *B. oleracea* is mainly controlled by QTL rather than major genes (Landry et al. 1992; Pang et al. 2018; Voorrips and Kanne 1997). One study reported that all of the cabbage and cauliflower accessions tested were completely susceptible to clubroot (Manzanares-Dauleux et al. 2000), but that report is not consistent with other published assessments. To date, 37 QTL/genes conferring resistance to *P. brassicae* have been identified on the C-genome of *B. oleracea* (Farid et al. 2020; Figdore et al. 1993; Grandclément and Thomas 1996; Landry et al. 1992; Peng et al. 2018; Voorrips and Kanne 1997). These include *CR2a, CR2, pb-3, pb-4, QTL1, QTL3, QTL9, Pb-Bo1, Pb-Bo2, Pb-Bo3, Pb-Bo4, Pb-Bo5a, Pb-Bo5b, Pb-Bo8, Pb-Bo9a, Pb-Bo9b, PbBo1, Pb-Bo (Anju) 1, Pb-Bo (Anju)2, Pb-Bo (Anju)3, Pb-Bo (Anju)4, CRQTL-YC, CRQTL-GN_1, CRQTL-GN_2, DIC.I-1, DIC.II-1, Rcr7, PbC4.1, PbC6, PbC7.1, PbC7.2, PbC8, PbC9.1, PbC3, PbC4.2, PbC7.3,* and *PbC9.2.* While a large number of *B. oleracea* genotypes have been screened for clubroot resistance (Carlsson et al. 2004; Crute et al. 1980; Manzanares-Dauleux et al. 2000; Voorrips and Kanne 1997), complete resistance has rarely been found. Crisp et al. (1989) evaluated more than 1000 *B. oleracea* accessions for clubroot resistance and found that some of the kale and Brussel sprouts carried moderate resistance. Most studies of the inheritance of clubroot resistance in *B. oleracea* have indicated that it is controlled by more than one gene (polygenic) (Laurens and Thomas 1993; Voorrips and Kanne 1997b). However, Dakouri et al. (2018) identified a major gene loci *Rcr7* in a cabbage

variety ‘Tekila’ for resistance to pathotype 3, the most abundant and virulent pathotype of *P. brassicae* on the Canadian Prairies prior to the introduction of CR canola.

1.2.9.3. Clubroot resistance in *B. napus*

Studies of the genetics of clubroot resistance in *B. napus* indicated that most of the major clubroot resistance genes in *B. napus* are found on the A-genome (derived from *B. rapa*), while most QTL conferring resistance are found on the C-genome (Neik et al. 2017; Piao et al. 2009; Rahman et al. 2014; Song et al. 2020;Diederichsen et al. 2006; Gustafsson and FALT 1986).

In early assessments, segregation analysis of *CR* genes from a resynthesized *B. napus* line indicated that clubroot resistance was controlled by the combined effect of at least two dominant, unlinked genes (Diederichsen and Sacristan 1996). These genes were assumed to be the same as those from European turnip (Bradshaw et al. 1997; Gowers 1982; Lammerink 1970) and the *B. rapa* ECD 04 (Frauen 1999; Gowers 1982). Ayers and Lelacheur (1972) also suggested that the genetic resistance in *B. napus* was controlled by one or two single independent genes from *B. rapa*. Manzanares-Dauleux et al. (2000) identified one major gene (*Pb-Bn1*) on chromosome A03 of *B. napus* ‘Darmor-bzh’ that conferred resistance to *P. brassicae* isolate Pb137-522. More recently, the *B. napus* cv. ‘Mendel’ was reported to carry one major gene and two recessive genes with efficacy against the widely distributed *P. brassicae* pathotypes in Europe (Diederichsen et al. 2006; Diederichsen et al. 2009). Furthermore, a single dominant *CR* locus effective against pathotypes 2, 3, 5, 6, and 8, classified according to Williams (1966), was identified on A08 chromosome of rutabaga, as well as the highly virulent pathotype 3, identified on chromosome A03 of ‘Mendel’ (Fredua-Agyeman & Rahman 2016; Hasan & Rahman 2016).

1.2.10. Genotyping-by-sequencing (GBS) and its application in mapping CR genes

Advances in next-generation sequencing (NGS) technologies for the sequencing of animal and plant genomes have helped to unlock the genetic properties of many species (Feuillet et al. 2011). These technologies have also been used to identify single nucleotide polymorphisms (SNPs), study genetic diversity, construct haplotype maps, and conduct genome-wide association studies (Metzker 2010). NGS has been used successfully to sequence and construct reference genomes of many important plant species, including *Arabidopsis thaliana* (Schranz and Mitchell-Olds 2006), *B. rapa* (Cai et al. 2017; Wang et al. 2011), *B. oleracea* (Liu et al. 2014; Parkin et al. 2014), *B. nigra* (Yang et al. 2016), *B. napus* (Chalhoub et al. 2014; Bayer et al. 2017, Song et al. 2020; Rousseau-Gueutin et al. 2020) and *B. juncea* (Yang et al. 2016). The availability of these genomes has enabled the identification of QTL and genes controlling yield, quality, and disease and pest resistance (Delourme et al. 2018). The NGS-based Brassica reference genomes have been used to identify genome-wide variants and genotype SNPs, construct high-resolution genetic maps, identify specific loci and predict candidate genes linked to the target loci (Yu et al. 2016; Yu et al. 2017; Dakouri et al. 2018; Pang et al. 2018). Identified SNPs can also be used to identify linkage disequilibrium (LD) between and among the loci in a population through GWAS and to accelerate the identification of candidate genes (Varshney et al. 2014) that are in LD in the population.

Genotype by sequencing technology is simple, quick, highly specific, reproducible, and applicable to genetically diverse species with large, complex genome, such as canola, to identify variants and explore genetic diversity. In GBS, genomic DNA is cut into thousands of small pieces using restriction enzymes or by sonication (ion torrent), and each DNA fragment is ligated to an adapter on each end (a short strand of DNA oligonucleotides that binds to the 5′ and 3′ end

of each fragmented DNA sequence in a sequencing library) to prepare the DNA libraries used for sequencing. Each sequence obtained from sequencing a DNA fragment + adapter (conducted in large batches called runs) is an individual read. The reads are processed through the removal of the adaptersand aligned to a pre-existing reference genome. Once aligned the software, SeqMan Pro 13 (DNASTAR, Madison, WI, USA), can quickly identify SNPs that represent small differences among isolates.

GBS technology has been used in GWAS to identify QTL or genes underlying traits in many species. GWAS of 472 *B. napus* accessions was used to identify nine QTL and 28 genes for clubroot resistance (Li et al. 2016). Similarly, GWAS was used to identify two resistance loci, *C09* and *A03b,* associated with the nitrogen effect on clubroot development in *B. napus* (Laperche et al. 2017). Dakouri et al. (2021) used GBS-GWAS to identify 13 important SNP loci that were associated with clubroot resistance against four major resistance-eroding pathotypes, 3A, 2B, 3D and 5X.

Lee and others (2016) used GBS technology to identify two major QTL (*CRQTL-GN_1* and *CRQTL-GN_2*) in *B. oleracea* that were associated with clubroot resistance based on a saturated genetic map of 4,103 SNPs from an F_2 population. Similarly, Yu et al. (2017) used GBS to identify and map three clubroot resistance loci, *Rcr4* on chromosome A03, *Rcr8* on A02, and *Rcr9* on A08, which were effective against pathotypes 2, 3, 5, 6, 8, and 5X of *P. brassicae*. Genotype by sequencing data obtained from a BC_1S_1 population developed from a cross between the *B. rapa* lines T19 (CR) and ACDC (susceptible) was used to construct the reference genome map to locate the genes. One thousand five hundred and eighty-four high-quality SNP variants were identified and used to construct a genetic map to identify and map the QTL conferring resistance to the pathotypes in the study (Yu et al. 2017).

1.2.11. Durable clubroot resistance in canola

The number of clubroot resistance genes in canola identified to date is limited, and the judicious deployment of these genes is important for the durability of CR canola. Gene pyramiding (the stacking of two or more resistance genes in the same variety) is a common and very effective *R*-gene deployment strategy (Lof and van der Werf 2017; Mundt 2014) and could extend the durability of clubroot resistance. The sequential use of *R*-genes (deployment of two single-gene resistant cultivars in a sequential way, where the second resistant variety comes into use after the resistance of the first resistant variety is overcome), as well as the simultaneous use of *R*-genes (two *R*-gene varieties deployed at the same time), could also contribute to prolonging the effectiveness of resistance in canola. Moreover, rotation of *R*-genes and a combination of all four strategies (gene pyramiding, sequential use of *R*-genes, *R*-gene rotation, and simultaneous use of resistant varieties), known as a 'mixed strategy', could help to protect the durability of the resistance genes (Lof and van Der Werf 2017; Dolatabadial et al. 2021). Such approaches could be combined with longer rotations out of CR canola to reduce selection pressure on *P. brassicae* populations or even the rotation of specific resistance genes in time or space. Regardless of the particular approach, the identification and mapping of major *R*-genes or QTL will become increasingly important for the sustainable production of canola.

1.2.12 Hypotheses and objectives

The research presented in this thesis was undertaken to test four main hypotheses:

1. Canola-quality *B. rapa* and *B. napus* do not possess resistance against some of the most important, virulent new pathotypes of *P. brassicae* identified in Alberta, Canada;
2. The clubroot-resistant *B. rapa* line ECD02 does not possess a resistance gene in its genome profile that is effective against the resistance-breaking pathotypes 3A, 3D, 3H, and 5X;

3. The *B. napus* cvs. 'AAFC695' and 'Mendel' do not possess resistance genes effective against the resistance-breaking pathotypes 3D, 3H, 5C, 5X, and 8J; and
4. Resistance to pathotypes 3A, 3D, 3H, and 5X in *B. rapa* is not controlled by a major gene; and the SNP markers developed to identify genes against pathotypes 3A, 3D, 3H, 5X, 5C, and 8J are not tightly linked.

From a more practical perspective, and given the development of genetic resources for resistance to clubroot in canola, these studies were undertaken with the following primary objectives:

1) To evaluate *B. napus* and *B. rapa* lines for resistance to the major new pathotypes of *P. brassicae* identified in Alberta;
2) To identify and map clubroot resistance genes in *B. napus* and *B. rapa* that were effective against the new pathotypes of *P. brassicae* through mapping by sequencing; and
3) To develop SNP markers that were tightly linked to clubroot resistance genes.

1.2.13. Figures

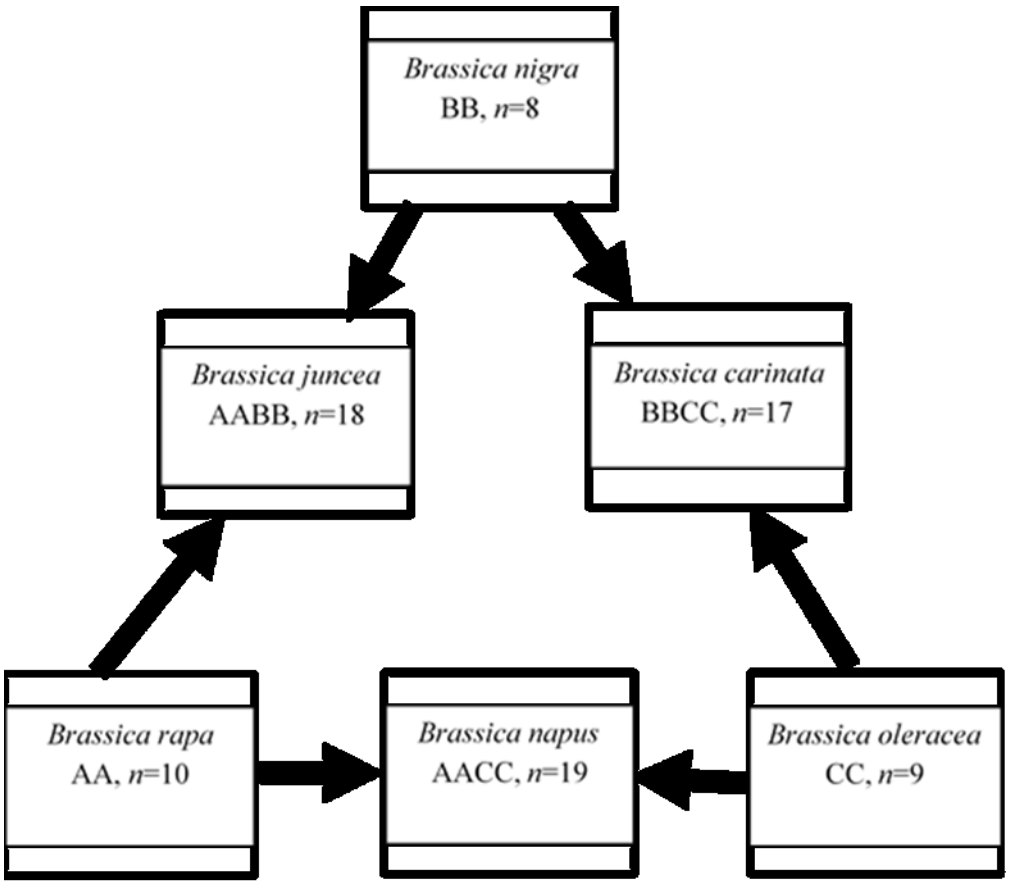


Figure 1. 1. The Triangle of U (1935), showing the genetic relationship among three diploid (*Brassica rapa, B. nigra*, and *B. oleracea*) and three amphidiploid *Brassica* species (*B. carinata*, *B. juncea,* and *B. napus*).

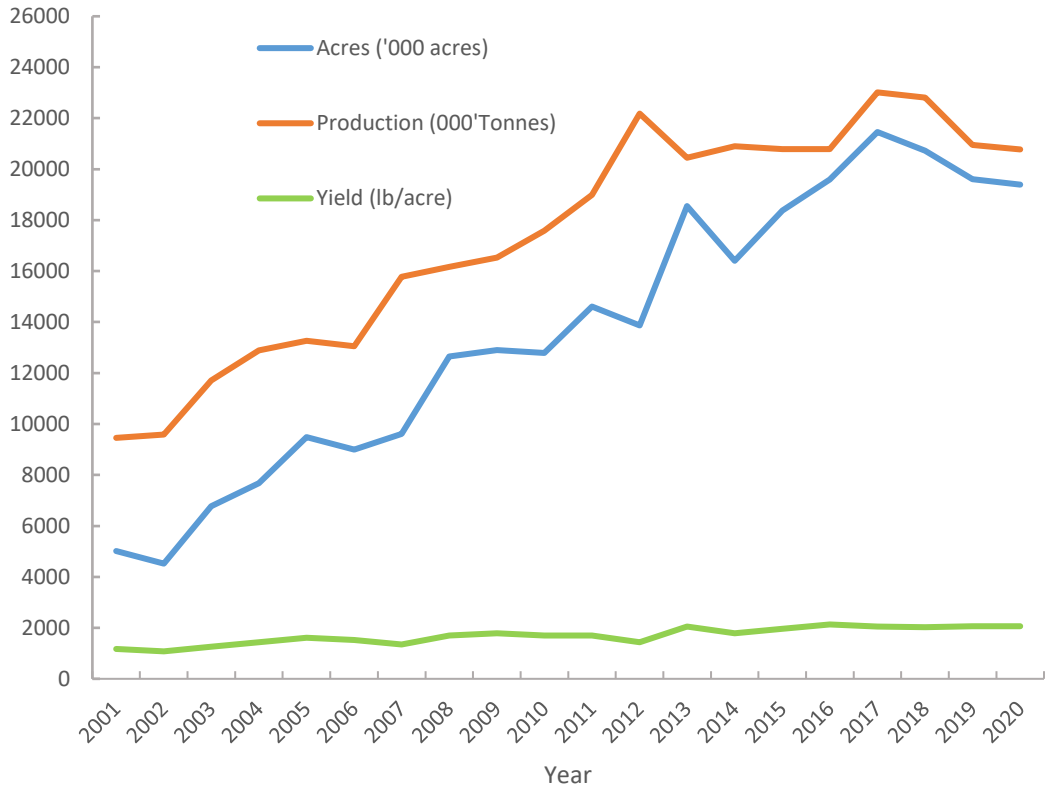


Figure 1. 2. Seeded acres, seed production, and yield of canola in Canada over time (source: Canola Council of Canada 2020).

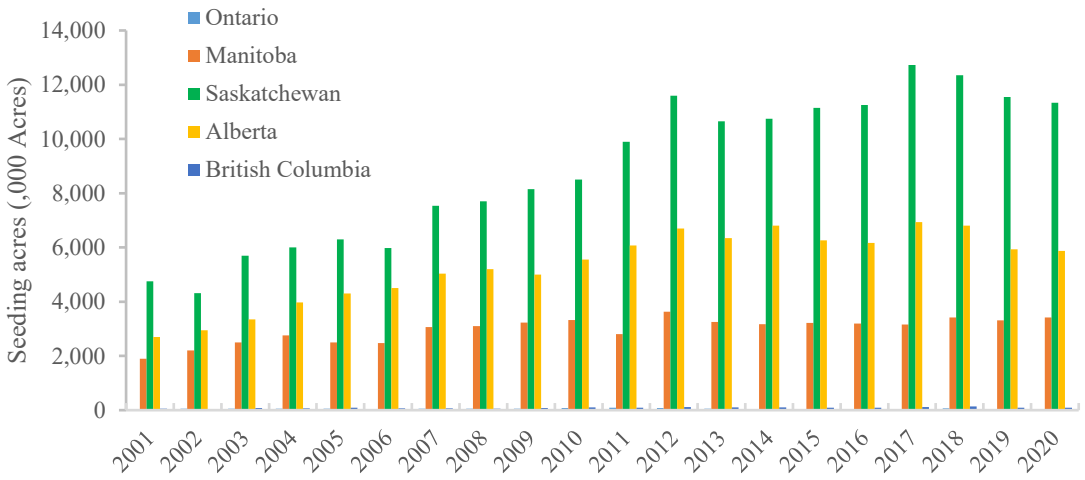


Figure 1. 3. Canola production across Canada (source: Canola Council of Canada 2020).

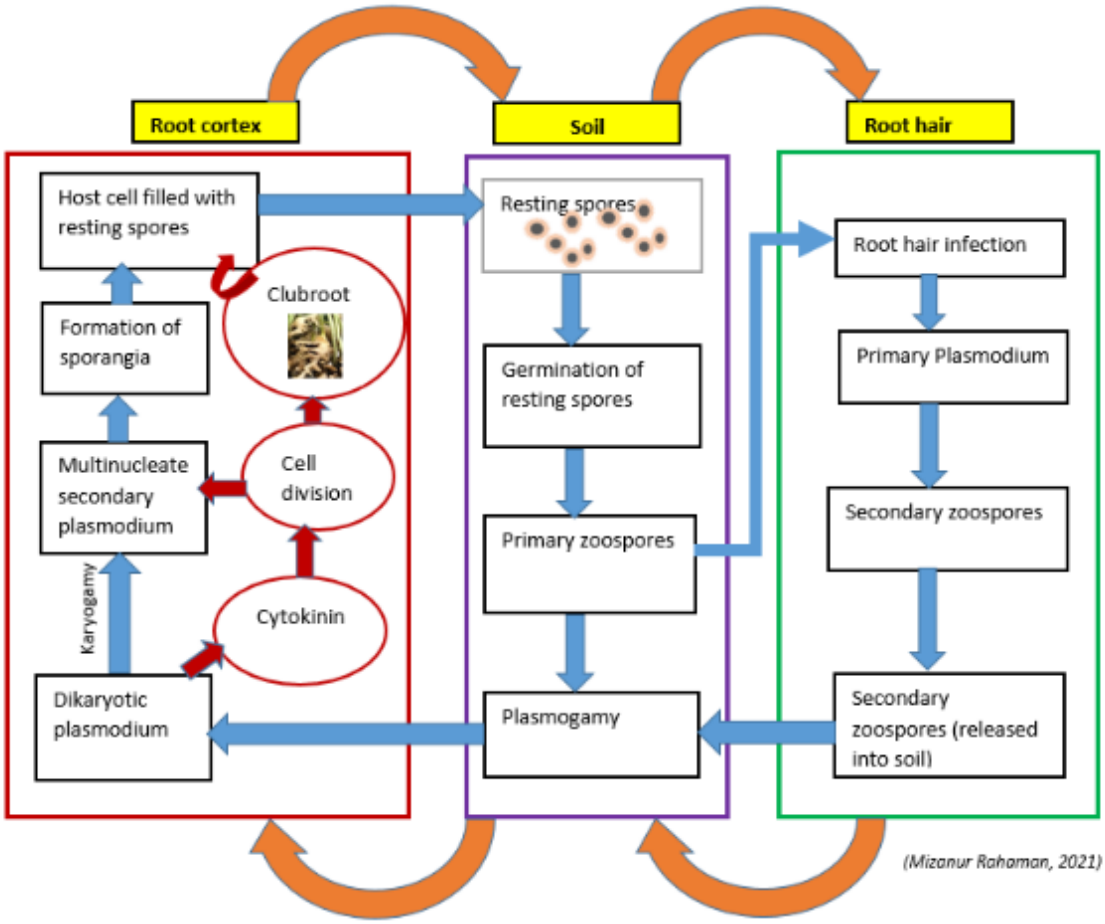


Figure 1. 4. Different stages of the *Plasmodiophora brassicae* life cycle (adapted from Ingram and Tommerup 1972; Kageyama and Asano 2009; Laila et al. 2020), from resting spore germination to infection, root gall development and pathogen multiplication in the roots.

Chapter 2. Evaluation of *Brassica napus* and *B. rapa* genotypes for reaction to resistance-breaking pathotypes of *Plasmodiophora brassicae*

2.1. Introduction

Clubroot disease of crucifers (family Brassicaceae) is caused by the soilborne obligate parasite *Plasmodiophora brassicae* (Woronin 1878). Infection of the roots of susceptible hosts results in the formation of large galls or clubs, which interfere with water and nutrient uptake and can lead to significant yield and quality losses (Strelkov & Hwang 2014; Laila et al. 2020). Clubroot has been spreading rapidly throughout *Brassica*-growing regions worldwide, with the occurrence of the disease confirmed in more than 60 countries (Dixon 2009). It is now one of the major and most devastating diseases of canola (rapeseed; *Brassica napus* L.) on the Canadian prairies (Tewari et al. 2005; Strelkov et al. 2018), where it causes yield losses of about 30%, although losses of up to 100% can occur under conditions favorable for the disease (Strelkov et al. 2007; Strelkov & Hwang 2014). The most effective and widespread strategy for clubroot management involves the planting of clubroot-resistant (CR) canola cultivars, which first became available in Canada in 2009 (Strelkov & Hwang 2014).

These CR cultivars show excellent resistance to many pathotypes of *P. brassicae*, including pathotype 3H (as defined on the Canadian Clubroot Differential (CCD) set; Strelkov et al. 2018), which was predominant on canola prior to the introduction of the resistance trait (Strelkov et al. 2006; Xue et al. 2008; Cao et al. 2009). Nonetheless, the resistance in most CR canola cultivars in Canada is believed to be based on a single-gene (Rahman et al. 2014; Peng et al. 2014), while the virulence of pathogen populations can shift quickly in response to the selection pressure imposed by resistant hosts (Diederichsen et al. 2003; LeBoldus et al. 2012).

As such, resistance-breaking pathotypes of *P. brassicae* were identified within 4 years of the introduction of CR canola (Strelkov et al. 2016). The first of the new, 'resistance-breaking' pathotypes was designated 5X on the CCD set (Strelkov et al. 2018); this pathotype is highly virulent on most CR canola. The identification of 5X and other resistance-breaking pathotypes, including 3A, 2B and 3D, has underscored the need to identify novel sources of resistance to *P. brassicae*.

About 74 clubroot resistance loci/genes have been identified in *B. rapa* (22)*, B. oleracea* (37) and *B. napus* (20) (Piao et al. 2009; Yu et al. 2016; Lee et al. 2016; Li et al. 2016; Chen et al. 2016), including the *Crr* and *CR* series in *B. rapa*, the *CR2*, *Pb*, and *Pb-Bo* series in *B. oleracea*, and the *Pb-Bn* and *PbBn* series in *B. napus* (Piao et al. 2009; Chen et al. 2013; Kato et al. 2013; Yu et al. 2016). This study was undertaken to evaluate a collection of *B. napus* and *B. rapa* lines for resistance to some of the major new pathotypes of *P. brassicae* identified on the Prairies in recent years, to help with the identification and mapping of clubroot resistance genes that could be used in rotations to manage and reduce selection pressure on *P. brassicae*.

2.2. Materials and Methods

2.2.1. Plant and pathogen material

A total of 50 *B. napus* and *B. rapa* lines/accessions were included in this study (Figure 2.2). These consisted of 34 *B. napus* lines resistant to *P. brassicae* pathotype 5X (Dakouri et al 2021) and 16 *B. rapa* lines (Tables 2.1- 2.2 and Tables S2.1-S2.2) resistant to pathotypes 3H or 5X (LG-2) (Yu et al. 2017). The *B. rapa* included 16 BC_1S_1 [ACDC × (ACDC × T19)] families developed from the *B. rapa* turnip breeding line T19 (Yu et al. 2017); these families carried three clubroot resistance loci (*Rcr4* for resistance to 3H, and *Rcr8* and *Rcr9* for resistance to 5X) individually or in combination. Seeds of the host genotypes were obtained from Agriculture and

Agri-Food Canada (AAFC), Saskatoon Research and Development Center (SRDC), Saskatoon, SK, or from the Plant Genetic Resources Centre (PGRC) of AAFC, Saskatoon, SK. In addition to the test genotypes, *B. rapa* subsp. *pekinenses* cv. 'Granaat' (European Clubroot Differential (ECD) 05), and two *B. napus* genotypes, 'Westar' and the line DH16516, were included as *P. brassicae*-susceptible checks.

Three field isolates of *P. brassicace*, representing pathotypes 3A (field isolate F3-14), 2B (F183-14), and 3D (F1-14) (Strelkov et al. 2018), were used to screen the *Brassica* families for clubroot resistance. Pathotype 3A is the predominant resistance-breaking pathotype in western Canada, while pathotype 3D is second-most common (Strelkov et al. 2018; Hollman et al. 2021). Pathotype 2B, while rare, has the widest virulence range among the hosts of the CCD set (Strelkov et al. 2018). The field isolates were maintained as frozen canola root galls, and were not multiplied further during the study to avoid shifts in the virulence of the inoculum.

2.2.2. Inoculum preparation

Inoculum was prepared from frozen root galls following Strelkov et al. (2006). Briefly, approximately 2.0-2.5 g of galled root tissue was ground in a mortar with a pestle in 50 ml of sterile, deionized distilled water (sdH_2O). The resulting homogenate was filtered through six layers of cheesecloth (American Fiber and Finishing Inc., Albemarle, North Carolina) into a glass beaker. The spore concentration was estimated with a hemocytometer (VWR, Mississauga, Ontario) and adjusted to 1×10^7 resting spores per mL^{-1} with sterile distilled water (Voorrips and Visser 1993). The inoculum was used immediately after its preparation as described below.

2.2.3. Inoculation

Seeds were surface-disinfected in 1% sodium hypochlorite for 1 min, washed 3-4 times with distilled water, and placed on wet Whatman No. 1 filter paper in Petri dishes. After 7 days,

the newly germinated seedlings were inoculated by dipping the roots in a freshly prepared *P. brassicae* resting spore suspension following Strelkov et al. (2016). The inoculated seedlings were then planted in 6 cm × 6 cm × 6 cm plastic pots filled with Sunshine LA4 potting mixture (Sunshine Growers, Vancouver, BC), at a density of one seedling per pot (Strelkov et al. 2016). To ensure successful inoculation, an additional 5 ml of inoculum solution was applied to the base of each seedling with a micropipette (Lamers and Toxopeus 1977). The seedlings were watered thoroughly and transferred to a greenhouse kept at 20 °C ± 2 °C with a 16 h photoperiod. The potting mixture was maintained at pH 6.5 for the first week after inoculation, with the pots kept in water-filled trays to ensure sufficient moisture for infection. The pots were then fertilized with 20 N:20 P:20 K and watered as needed for the proper growth and development of the seedlings (Strelkov et al. 2016). Each treatment was replicated three times, with 12 pots per replicate. Experiments were arranged in a completely randomized design (CRD).

2.2.5. Disease assessment

The seedlings were maintained in the greenhouse for six weeks, at which point they were carefully removed from the potting mix and the roots were washed under running water. The roots were then scored individually for clubroot symptom severity on a 0-3 scale (Kuginuki et al. 1999), where: 0 = no galls, 1 = a few small galls, 2 = moderate galls, and 3 = severe galling on the roots. A disease severity index (DSI) was calculated for each replicate using the formula of Horiuchi and Hori (1980) as modified by Strelkov et al. (2006):

$$\text{DSI (\%)} = \frac{\sum(n \times 0 + n \times 1 + n \times 2 + n \times 3)}{\text{N} \times 3} \times 100\%$$

Where, *n* indicates the number of plants in each symptom severity class, N is the total number of plants, and 0, 1, 2, and 3 are the symptom severity classes. A mean DSI ± standard error (SE) was calculated across replicates for each treatment (host genotype/pathotype combination), with

a DSI <60% regarded as resistant (R) and a DSI ≥60% regarded as susceptible (S) (Yu et al. 2017; Yu et al. 2021).

2.3. Results

2.3.1. Reaction of *B. rapa* lines

None of the 16 *B. rapa* families evaluated were resistant to pathotypes 3A, 2B, or 3D (Table 2.1, and Table S2.1). The DSI on the host lines ranged between 91.2% ± 8.8% to 100% ± 0.0% (Table 2.1). In response to inoculation with pathotype 3A, the DSI ranged from 97.2% to 100%. Similarly, in response to inoculation with pathotype 2B, the DSI ranged from 91.2% to 100%. In response to inoculation with pathotype 3D, all of the tested families developed a DSI = 100%, with the exception of T19-X79 which had an ID = 97.2% ± 2.8%. As expected, the susceptible *B. rapa* check, ECD 05, developed a DSI = 100% ± 0.0% against each of pathotypes 3A, 2B, and 3D.

2.3.2. Reaction of *B. napus* lines

Among the 34 *B. napus* accessions tested, three (CGN06902, CGN17369, and AAFC695) were found to be highly resistant to all three of the pathotypes 3A, 2B, and 3D, with DSI values ranging between 0% ± 0.0% and 13.9% ±2 .8% (Table 2.2, Figure 2.1, and Table S2.2). The accession CN46235 was resistant to pathotypes 3A (DSI = 21.3% ± 3.7%) and 3D (DSI = 19.4% ± 13.9%), but susceptible to pathotype 2B (DSI = 46.3% ± 0 .0%). Similarly, accession PI 284859 also showed a differential reaction to the pathotypes; it was resistant to pathotype 3D (DSI = 11.1%±8.4%), but susceptible to 3A and 3D (DSI values of 56.5% ± 13.0% and 100 ± 0.0%, respectively). The remaining 29 *B. napus* accessions were susceptible or highly susceptible to pathotypes 3A, 2B and 3D, with DSI values ranging from 57% to 100% (Table

2.2). The susceptible *B. napus* checks 'Westar' and line DH16516 developed a DSI = 100% ± 0.0% in response to all pathotypes.

2.4. Discussion

The *B. rapa* genotypes represented BC_1S_1 families developed from the *B. rapa* line T19, which was also included in the current study. None of the BC_1S_1 families nor line T19, however, showed any resistance to pathotypes 3A, 2B or 3D. Yu et al. (2017) identified and mapped a major QTL *Rcr4* on chromosome A03 of this line, which was effective against the 'original' pathotypes 2, 3, 5, 6, and 8 (Williams 1966), which correspond to the CCD pathotypes 2F, 3H, 5I, 6M and 8N, respectively, and cannot overcome 'Mendel' type resistance (Strelkov et al. 2018). Two other QTL, *Rcr8* and *Rcr9* on chromosomes A02 and A08 and conferring resistance to pathotype 5X, were also mapped from the population developed from T19 (Yu et al. 2017). Given the high degree of susceptibility observed in T19 and all of the BC_1S_1 families in this study, it appears that *Rcr4, Rcr8* and *Rcr9* are not effective against pathotypes 3A, 2B and 3D.

More promising results were obtained with some of the *B. napus* accessions. The accessions CGN06902, CGN17369, and AAFC695were found to be highly resistant to *P. brassicae* pathotypes 3A, 2B and 3D. Many studies have examined the genetics of clubroot resistance in *B. napus* (Ayers and Lelacheur 1972; Gustafsson and Fält 1986; Bradshaw et al. 1997; Manzanares-Dauleux et al. 2000; Diederichsen et al. 2006; Hasan & Rahman 2016; Peng et al. 2014; Fredua-Agyeman et al. 2020), which has been found to be mediated mostly by genes on the A-genome (Gustafsson & Fält 1986; Diederichsen et al. 2006). Indeed, CR germplasm has been identified from *B. napus* genetic backgrounds and used as a genetic resource for the development of *Brassica* varieties with resistance against multiple pathotypes of *P. brassicae* (Dakouri et al. 2021; Liu et al. 2018; Peng et al. 2014; Hasan et al. 2012). Clubroot resistance in

B. napus is mostly oligogenic (Crute et al. 1980), with more than one gene responsible and complex genetic segregation. Further studies are planned to identify and map the CR genes present in the resistant *B. napus* genotypes identified in this study. Ultimately, the identification of host genotypes carrying effective resistance against 3A, 2B, and 3D will be critical for the sustainable management of clubroot of canola.

2.5. Tables

Table 2. 1. Clubroot Disease Severity Index (DSI) on *Brassica rapa* genotypes following inoculation with field isolates representing pathotypes 3A, 2B and 3D of *Plasmodiophora brassicae*.

Genotype	Species	Type	CR loci/genes present	Host reaction to each pathotype*		
				3A	2B	3D
				DSI(%)	DSI(%)	DSI(%)
T19-X17	*B. rapa*	BC_1S_1	*Rcr4,Rcr8,Rcr9*	97.2±2.8	95.6±4.4	100±0.0
T19-X27	*B. rapa*	BC_1S_1	*Rcr4,Rcr9*	100±0.0	98.9±1.1	100±0.0
T19-X28	*B. rapa*	BC_1S_1	*Rcr4,Rcr8,Rcr9*	98.1±1.9	96.3±2.7	100±0.0
T19-X33	*B. rapa*	BC_1S_1	*Rcr8,Rcr9*	100±0.0	100±0.0	100±0.0
T19-X38	*B. rapa*	BC_1S_1	*Rcr4,Rcr9*	99.8±0.2	100±0.0	100±0.0
T19-X46	*B. rapa*	BC_1S_1	*Rcr4,Rcr8*	99.8±0.2	98.9±1.1	100±0.0
T19-X59	*B. rapa*	BC_1S_1	No	100±0.0	97.8±2.2	100±0.0
T19-X65	*B. rapa*	BC_1S_1	*Rcr9*	97.2±2.8	93.3±6.7	100±0.0
T19-X66	*B. rapa*	BC_1S_1	*Rcr8*	100±0.0	100±0.0	100±0.0
T19-X71	*B. rapa*	BC_1S_1	*Rcr4,Rcr8*	97.8±2.2	100±0.0	100±0.0
T19-X78	*B. rapa*	BC_1S_1	*Rcr4*	100±0.0	91.2±8.8	100±0.0
T19-X79	*B. rapa*	BC_1S_1	*Rcr9*	97.2±2.8	100±0.0	97.2±2.8
T19-X80	*B. rapa*	BC_1S_1	*Rcr4*	100±0.0	100±0.0	100±0.0
T19-X82	*B. rapa*	BC_1S_1	*Rcr8*	100±0.0	100±0.0	100±0.0
T19-X84	*B. rapa*	BC_1S_1	*Rcr8,Rcr9*	100±0.0	100±0.0	100±0.0
T19-X92	*B. rapa*	BC_1S_1	No	100±0.0	100±0.0	100±0.0
ECD 05**	*B. rapa*	Cultivar	No	100±0.0	100±0.0	100±0.0

*Values represent the mean ± standard error across replicates
**Susceptible check, European Clubroot Differential (ECD) 05

Table 2. 2. Clubroot Disease Severity Index (DSI) on *Brassica napus* genotypes following inoculation with field isolates representing pathotypes 3A, 2B and 3D of *Plasmodiophora brassicae*.

Genotype	Species	Type	Host reaction to each pathotype*		
			3A	2B	3D
			DSI%	DSI%	DSI%
ECD 06	*B. napus*	Oilseed rape	93.5±4.5	83.3±16.7	100±0.0
ECD 09	*B. napus*	Oilseed rape	71.2±6.4	100±0.0	100±0.0
DH6756-5	*B. napus*	Oilseed rape	100±0.0	100±0.0	100±0.0
CN31153	*B. napus*	Rutabaga	100±0.0	100±0.0	100±0.0
CN31154	*B. napus*	Rutabaga	100±0.0	88.9±11.1	100±0.0
CN31304	*B. napus*	Rutabaga	97.2±2.8	95.4±6.6	100±0.0
CN31391	*B. napus*	Rutabaga	57.4±6.5	71.3±28.7	97.2±2.8
CN31403	*B. napus*	Rutabaga	100±0.0	100±00	100±0.0
CN31417	*B. napus*	Rutabaga	89.8±11.2	100±0.0	100±0.0
CN31450	*B. napus*	Rutabaga	100±0.0	95.8±4.2	97.2±2.8
CN31451	*B. napus*	Rutabaga	100±0.0	100±0.0	100±0.0
CN31452	*B. napus*	Rutabaga	100±0.0	100±0.0	100±0.0
CN31454	*B. napus*	Rutabaga	98.1±1.9	100±0.0	100±0.0
CN31457	*B. napus*	Rutabaga	59.6±7.0	91.7±8.2	84.3±15.7
CN35993	*B. napus*	Oilseed rape	100±0.0	100±0.0	97.2±2.8
CN39440	*B. napus*	Oilseed rape	85.2±14.8	100±0.0	74.5±17.2
CN39441	*B. napus*	Oilseed rape	100±0.0	100±0.0	100±0.0
CN39443	*B. napus*	Oilseed rape	97.0±3.0	100±0.0	100±0.0
CN43206	*B. napus*	Oilseed rape	91.1±9.9	100±0.0	100±0.0
CN46235	*B. napus*	Oilseed rape	21.3±3.7	46.3±0.0	19.4±13.9
Ames1669	*B. napus*	Oilseed rape	100±0.0	100±0.0	100±0.0
Ames6075	*B. napus*	Oilseed rape	97.0±3.0	100±0.0	100±0.0
PI284859	*B. napus*	Oilseed rape	56.5±13.0	100±0.0	11.1±8.4
PI305280	*B. napus*	Oilseed rape	100±0.0	100±0.0	97.2±2.8
PI311727	*B. napus*	Oilseed rape	97.2±2.8	100±0.0	95.4±14.6
CGN06822	*B. napus*	Oilseed rape	100±0.0	100±0.0	100±0.0

Genotype	Species	Type	Host reaction to each pathotype*		
			3A	2B	3D
			DSI%	DSI%	DSI%
CGN06902	*B. napus*	Oilseed rape	4.3±1.23	0±0.0	13.9±2.8
CGN07237	*B. napus*	Oilseed rape	100±0.0	100±0.0	100±0.0
CGN06896	*B. napus*	Oilseed rape	99.1±0.9	100±0.0	100±0.0
CGN17369	*B. napus*	Oilseed rape	0±0.0	0±0.0	0±0.0
AAFC695	*B. napus*	Oilseed rape	6.48±4.6	3.7±7.3	4.6±6.5
CGN17381	*B. napus*	Oilseed rape	98.0±2.0	100±0.0	100±0.0
CGN13919	*B. napus*	Oilseed rape	100±0.0	100±0.0	100±0.0
Laurentian	*B. napus*	Rutabaga	100±0.0	100±0.0	100±0.0
DH16516**	*B. napus*	Oilseed rape	100±0.0	100±0.0	100±0.0
Westar**	*B. napus*	Oilseed rape	100±0.0	100±0.0	100±0.0

*Values represent the mean ± standard error across replicates

**Susceptible checks

2.6. Figures

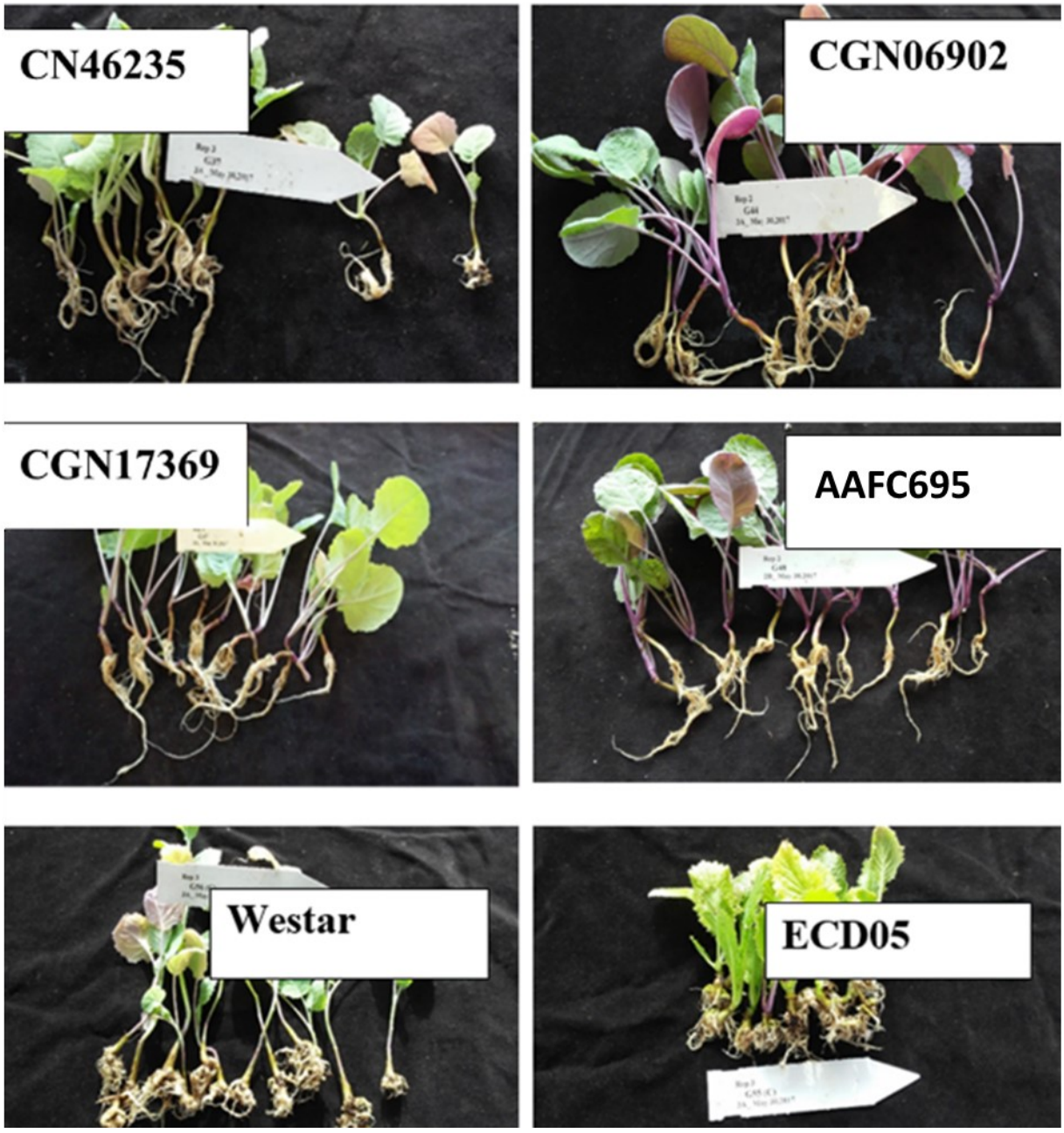


Figure 2. 1. Four resistant *Brassica napus* genotypes, CN46235, CGN06902, CGN17369, and AAFC695, were identified in an evaluation of 50 *B. rapa* and *B. napus* genotypes tested against pathotypes 3A, 2B, and 3D of *Plasmodiophora brassicae* under greenhouse conditions. Symptom development on the susceptible checks, *B. napus* 'Westar' and *B. rapa* European Clubroot Differential (ECD) 05, is shown for comparison.

Figure 2. 2. Photograph showing the experimental set-up during phenotyping of 50 *Brassica rapa* and *B. napus* genotypes for resistance to pathotypes 3A, 2B, and 3D of *Plasmodiophora brassicae*.

Chapter 3. Identification of a major gene for resistance to four pathotypes of *Plasmodiophora brassicae* in *Brassica rapa* turnip ECD02

3.1. Introduction

Brassica rapa (genome designated as AA, 2*n* = 20) is one of the diploid progenitors of *B. napus* (AACC, 2*n* = 38) (U, 1935), a species which plays a significant role in vegetable oil production in Canada and worldwide. The A genome also occurs in *B. juncea* (AABB, 2*n* = 36) (U, 1935), contributing to the genetic diversity of these amphidiploid species (Chen et al. 2013; Qian et al. 2006). *B. rapa* is thought to have originated in the Mediterranean region and gradually spread to Scandinavia, Central Europe, Japan, China, and India (Prakash et al. 1980; Gomez-Campo, 1999).

Clubroot disease caused by the obligate parasite *Plasmodiophora brassicae* Woronin is a threat to canola production in Canada (Strelkov and Hwang, 2014), where it can cause average yield losses of 10-15% (Dixon 2009; Botero-Ramirez et al. 2022) and losses as high as 30-100% in severe infections (Tewari et al. 2005; Strelkov et al. 2007a). Clubroot was first identified on canola in Alberta in 2003 (Tewari et al. 2005) and is spreading rapidly across the Canadian Prairies (Gossen et al. 2015; Hollman et al. 2021). As a result, clubroot represents an important potential constraint to canola production in Canada and could have a significant economic impact, as this crop is valued at $15.4 billion (CDN) annually (Rempel et al. 2014). Clubroot also occurs in many other regions of the world, including Australia, southeast Asia, northern and central Europe, the USA, and Latin America (Donald, 2005; Yang et al. 2012; Tanaka et al. 2001; Diederichsen et al. 2014; Wallenhammar, 1996; Howard et al. 2010; Chittem et al. 2014; Botero-Ramirez et al. 2019).

The release of the first clubroot-resistant (CR) canola cultivar, '45H29'(Pioneer Hi-Bred, ON, Canada), in Canada in 2009 (Strelkov et al. 2018) was quickly followed by other CR cultivars with a similar resistance profile (Deora et al. 2012). In 2013, the breakdown of the existing clubroot resistance by a new and virulent pathotype, named pathotype 5X, was confirmed in Canada near Edmonton, Alberta (Strelkov et al. 2018). The identification of additional new virulent pathotypes that could not be characterized using the existing differential systems led to the development of a new differential system known as the Canadian Clubroot Differential (CCD) set (Strelkov et al. 2018). Using the CCD, a number of new virulent pathotypes of *P. brassicae* have been identified in Canada (Strelkov et al. 2018; Hollman et al. 2021). Many of these pathotypes, including 3A, 3D and 5X, are highly virulent on the initial group of CR canola cultivars (Strelkov et al. 2016; Strelkov et al. 2018). Researchers across the region are working to develop durable clubroot management strategies to reduce the losses caused by these new pathotypes.

Soil amendments (Wellman,1930; Campbell et al. 1989; Donald & Porter, 2014; Fox et al. 2021), cultural practices (Dixon, 1991; Murakami et al. 2002; McDonald et al. 2004; Tremblay et al. 2005; Ahmed et al. 2011; Donald & Porter, 2014), fungicides and fumigants (Naiki and Dixon, 1987; Hwang et al. 2011; Peng et al. 2014; Donald and Porter, 2014) as well as biological control methods (Peng et al. 2014) have been all assessed for their efficacy against clubroot. However, the planting of CR cultivars remains the most effective, inexpensive and environmentally friendly approach (Rahman et al. 2014; Peng et al. 2014; Strelkov et al. 2016), which is why it has become the most commonly used clubroot management strategy in Canada.

After infection of the root hairs by the pathogen, resistant host plants initially show basal resistance (Dekhuijzen, 1979; Fuchs and Sacristan, 1996; Kobelt et al. 2000; Gravot et al.

2011; Lahlali et al. 2017) to all pathotypes, followed by race or pathotype-specific resistance (*R-gene*-mediated resistance) (Crute et al. 1980; Rahman et al. 2014; Peng et al. 2014). More than 20 CR loci, including about eight major genes and 12 QTL, have been identified and mapped (Sakamoto et al. 2008; Diederichsen et al. 2009; Piao et al. 2009; Hatakeyama et al. 2017; Yu et al. 2017; Karim et al. 2020) in the A genome against pathotypes of *P. brassicae*. Most of the CR loci have been identified on chromosome A03, including *CRa* (Matsumoto et al. 1998), *CRb* (Piao et al. 2004), *CRd* (Pang et al. 2018) *CRk* (Sakamoto et al. 2008), *Crr3* (Hirai et al. 2004; Saito et al. 2006), *PbBa3.1* to *PbBa3.2* (Chen et al. 2013), *Rcr1* (Chu et al. 2014), *Rcr2* (Huang et al. 2017), *Rcr4* (Yu et al. 2017), and *Rcr5* (Huang et al. 2019). Others have been mapped to chromosome A08, including *Crr1* (Suwabe et al. 2003), *CRs* (Laila et al. 2019), *Rcr9* (Yu et al. 2017), *Rcr3* (Karim et al. 2020) and $Rcr9^{wa}$ (Karim et al. 2020), chromosome A02, including *CRc* (Sakamoto et al. 2008) and *Rcr8* (Yu et al. 2017), and chromosomes A01 (*Crr2*; Suwabe et al. 2006) and A06 (*Crr4*; Suwabe et al. 2006).

Vegetable turnips (*B. rapa* subsp. *rapifera*) have proven to be one of the best sources for identifying CR genes effective against many pathotypes of *P. brassicae* in Canada and across the world. Several turnip lines have been included in the European Clubroot Differential (ECD) set for differentiating pathotypes of *P. brassicae* (Buczacki et al. 1975; Jones et al. 1982). One of these turnip lines, ECD02, is also included in the CCD set. ECD02 was resistant to all 36 pathotypes identified in Canada to date (Strelkov et al. 2018; Askarian et al. 2021; Hollman et al. 2021). Therefore, this genotype represents a very important source for developing canola cultivars with resistance to clubroot in Canada.

The *CRa* resistance gene, originating from ECD02 (reviewed by Piao et al. 2009) and providing resistance to Japanese isolates of *P. brassicae*, was identified (Matsumoto et al. 1998)

in Chinese cabbage (*B. rapa* subsp. *pekinensis*) and located on chromosome A03. Molecular markers linked to *CRa* were developed (Matsumoto et al. 2005) and the gene, which encodes a toll-interleukin-1 receptor, nucleotide binding site and leucine-rich repeat (TIR-NBS-LRR, TNL) protein (Ueno et al. 2012), was isolated from the Chinese cabbage donor. A study of resistance to the Canadian pathotypes 3H, 5X, and 5G confirmed the presence of *CRa* and *Crr1* in the ECD02 F2 population, based on phenotypic and molecular data, and resistance/susceptibility segregation ratios (Fredua-Agyeman et al. 2020). Two genes for resistance to a field isolate (likely pathotype 3H) of *P. brassicae*, *BraA.CR.a* (A03) and *BraA.CR.b* (A08), were also identified in ECD02 (Hirani et al. 2018). However, no research on the identification and mapping of resistance genes to important new pathotypes in ECD02 has been reported.

The current study was undertaken to identify and map CR genes/loci in *B. rapa* ECD02 with efficacy against the most prevalent pathotypes (3A, 3D, 3H, and 5X) of *P. brassicae* on the Prairies, using genotyping-by-sequencing (GBS), QTL analysis and conventional linkage mapping approaches. Potential candidate gene(s) in the target region were identified and bulked segregant analysis (BSA) was performed to identify SNP markers and develop markers tightly linked to the gene(s). The mapping of gene(s) effective against these important new pathotypes will assist breeders in developing Brassica crop cultivars for effective clubroot management.

3.2. Materials and Methods

3.2.1. Parental lines and development of BC_1/BC_1S_1 population

Seed of ECD02, a turnip (*B. rapa*) cultivar, was provided by Dr. G. R. Dixon (The University of Warwick, Wellesbourne, Warwick, UK). ECD02 was crossed with a highly susceptible *B. rapa* line, ACDC, which was a self-compatible, doubled haploid (DH) line developed by Dr. K. Falk at the Saskatoon Research and Development Centre (SRDC),

Agriculture and Agri-Food Canada (AAFC). ECD02 is a winter-type vegetable, so vernalization was needed to induce flowering. After 8 weeks of vernalization (4°C;16 hr light/ 8 hr dark), the plants were brought back to the greenhouse (21˚C/18˚C) to flower. The plants were covered with a transparent plastic pollination bag at the bud initiation stage to exclude pollen from other plants. Line ACDC was also planted in the greenhouse and allowed to flower, with each flower cluster covered with a pollination bag to prevent cross-pollination. The F_1 population was produced by crossing the lines ACDC × ECD02 where the line ECD02 served as a pollen donor. The resulting F_1 plants were backcrossed with line ACDC to develop a BC_1 population using the same protocol as for the initial cross. The F_1 plants were vernalized for 8 weeks as described previously. Some of BC_1 lines did not produce buds until 6 weeks after planting, so those plants were vernalized at 4°C and 16 hr light / 8 hr dark for 8 weeks. All the BC_1 lines were selfed by covering each plant with a plastic pollination bag to produce a BC_1S_1 population. The BC_1 were self-incompatible, so a 3% NaCl solution was sprayed to each of the BC_1 lines to overcome the self-incompatibility barrier (Yu et al. 2017). This provided a sufficient amount of seed from each plant for phenotyping against the selected pathotypes of *P. brassicae*. Ninety-three BC_1S_1 lines were developed.

3.2.2. Evaluating reaction to pathotypes

Seed from the 93 BC_1S_1 lines and the parental lines (ECD02 and ACDC) was used to assess the reaction to four important *P. brassicae* pathotypes from Canada, 3A, 3D, 3H, and 5X (field isolate F.3-14 for pathotype 3A; field isolate F.1-14 for 3D; field isolate P.41-14 for 3H; and field isolate LG02 for 5X).

A universally susceptible line, DH16516, originating from the *B. napus* cultivar ‘Topas’, which was provided by Dr. G. Séguin-Swartz at AAFC, SRDC, Saskatoon, was included as a

susceptible check. A DH line, NRC11-24, developed by Nutrien Ag Solutions (Saskatoon, SK) and with known resistance to the pathotype 3H, but susceptible to 3A, 3D, and 5X, was used as a second susceptible check to verify the pathogenicity of pathotypes 3A, 3D, and 5X, and as a resistant check for 3H. Two DH lines (AAFC-Y12 and AAFC-Y68) developed at AAFC, Saskatoon, which carry the resistance loci *Rcr9*^{ECD01} and *Rcr10*^{ECD01} and are highly resistant to pathotypes 3A, 3D, 3H and 5X (Yu et al. 2021), were included as resistant controls.

Twelve seedlings of each plant, including the parents, were inoculated individually with each of the four pathotypes under controlled conditions in a growth chamber. The inoculum suspension was prepared following a standard protocol (Strelkov et al. 2016) modified by Karim et al. (2020). Briefly, frozen galls were cut into small pieces and soaked in water in a glass beaker for 20–30 min, then homogenized in a blender for 2–3 min and strained through two layers of nylon cloth. The spore concentration was estimated using a hemocytometer, adjusted to 1×10^7 resting spores mL^{-1}, as described in Chapter 2, and stored at -20°C until required.

Seedlings of each plant were grown in 10 cm × 10 cm plastic pots filled with a commercial soil-less mix (Sunshine Mix 3, TerraLink Horticulture Inc. BC, Canada), packed and set in a plastic tray to catch and contain any runoff, with 12 seedlings per pot and 32 pots per tray. The pots were thoroughly soaked with water, allowed to drain and excess water was removed. After seeding, each tray was covered with a transparent plastic dome in a growth chamber set at 21°C ± 2°C under 16 hr light / 8 hr dark for one week. At one week after planting, the seedlings were inoculated with 15 ml of spore solution per pot. Immediately after inoculation, the trays were covered again and moved back to the growth chamber. Watering of the seedlings and trimming of leaves were carried out as needed. At 5 weeks after inoculation, the plants were uprooted, washed under tap water, and scored using the 0–3 rating scale

described by Kuginuki et al. (1999), where: 0 = no club symptoms on roots, 1 = a few small clubs, 2 = moderate clubbing, and 3 = severe clubbing on the whole roots (Figure S3.1). Clubroot severity was calculated for each line as a Disease Severity Index (DSI) following Horiuchi and Hori (1980) as modified by Strelkov et al. (2006), based on the following formula:

$$\text{DSI (\%)} = \frac{\sum(n \times 0 + n \times 1 + n \times 2 + n \times 3)}{\text{N} \times 3} \times 100\%$$

Where DSI is the overall severity on a line; *n* is the number of plants in each class; N is the total number of plants assessed; and 0, 1, 2 and 3 are the disease severity classes.

The lines from the BC_1S_1 population were separated into two categories: resistant (R) or susceptible (S). A line was regarded as R when the DSI < 60% and susceptible when the DSI ≥ 60% (Yu et al. 2017). The segregation ratio of R and S was calculated and goodness of fit was tested with a $\chi 2$ test using Microsoft Excel. In addition, the correlation coefficients among DSI values of the tested plants were calculated and the significance of the correlation was tested with a t-test (Iversen et al. 1997) using Microsoft Excel. The study was repeated and the results of the second repetition were used to identify and map QTL from the population

3.2.3. DNA extraction, sequencing and alignment of short reads to a reference genome

DNA was extracted from young leaf tissues of the 93 BC_1 lines and the parental lines using QIAGEN DNeasy kits following the manufacturer's directions. The quality of the extracted DNA and concentration was evaluated with Qubit Fluorometer, NanoDrop (Thermo Scientific™) and Microplate Reader and sample integrity was confirmed by gel electrophoresis, and the amount of DNA was adjusted to provide high-quality sequences from each sample. Genotyping-by-sequencing (GBS) was performed on an Illumina platform with pair-end sequencing at BGI Americas Corp (Cambridge, MA, USA). Whole genome sequencing of the parent lines ECD02 and ACDC was performed at the Plant Biotechnology Centre (Saskatoon,

SK, Canada). A DNA library was constructed following the protocol of Dakouri et al. (2018). The complexity of the extracted DNA was reduced by digesting the DNA with a methylation-sensitive restriction enzyme ApeKI. The fragments produced from the enzymatic digestion were ligated to enzyme-specific adapters and amplified by PCR. The ApeKI-GBS libraries were constructed, and 100-bp long GBS reads generated. The short readssequences obtained from the two parental lines and 93 BC_1 lines were aligned using SeqMan NGen 16 (DNASTAR, Madison, WI) against the *B. rapa* reference genome v3.0 (Chiifu) downloaded from http://brassicadb.org/brad/downloadOverview.php (Zhang et al. 2018).

3.2.4. SNP filtering, linkage map construction and QTL detection

The assembled and aligned sequences were analyzed using ArrayStar 16 (DNASTAR) to identify SNP variants in the sequences when compared with the *B. rapa* reference genome v3.0. The sequences of the 93 BC_1 lines were filtered to leave only high-quality SNPs; the variants were reduced by at least 50% based on the SNP criteria of depth coverage > 5, quality score (Q) >30, and the SNP percentage > 10 on the ArrayStar platform. The remaining SNPs were further filtered on JoinMap 4.1 to select tightly linked SNPs, and a linkage map was drawn using Mapchart 2.1 (Voorrips, 2002) using only SNPs with < 20 cM distance; SNPs > 20 cM were not regarded as tightly linked, and hence not used, as there would be a chance of recombination, between the adjacent markers. The GBS-SNP sites were named based on the chromosome name and position of the SNPs on the chromosome sequence on the reference genome (CF: 'Chiifu'). SNP loci from the R parent (ECD02) were scored as 'H' and those from the susceptible parent (ACDC) as 'A'. The filtered SNPs were used in association with the phenotypic data to map QTL associated with the clubroot-resistance trait using the IciMapping Inclusive Composite Interval Mapping (ICIM) method (Meng et al. 2015). IciMapping was run at 1,000-permutations

(known as bootstrapping) with a type I error rate of 0.01 for QTL declaration. The QTL identified in this analysis were authenticated based on a maximum LOD value with the phenotypic variation (%PVE), additive (Add) effect of the QTL and confidence interval (CI) on the trait of interest.

3.2.5. Bulked segregant analysis (BSA) and Kompetitive Allele Specific PCR (KASP)

The phenotype (R or S) of each BC_1 plant was determined, based on the mean DSI against each of the four pathotypes (3A, 3D, 3H and 5X) assessed on the BC_1S_1 population. As noted above, since the lines in the BC_1 population are heterozygous, a line was regarded as R when the DSI < 60% and susceptible when the DSI ≥ 60% (Yu et al. 2017). An R plant was scored as 'H' and an S plant as 'A'. Linkage analysis was performed with the SNP markers and phenotypes using JoinMap 4.1 (Van Ooijen, 2001).

To identify SNP sites tightly linked to the QTL and develop robust SNP markers for use in marker-assisted selection, 43 resistant BC_1 lines were combined to form an R bulk and 50 susceptible BC_1 lines to form an S bulk, and the GBS short reads from each bulk were then aligned with the *B. rapa* 'Chiifu' reference genome using SeqMan NGen 16. SNP genotyping was conducted 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 were determined based on the mean DSIs of the four pathotypes using JoinMap 4.1 (Van Ooijen, 2001).

3.2.6. Identification of genes encoding disease resistance proteins in the target region

Genes encoding disease resistance proteins, Receptor-like protein (RLP), Receptor-like kinase (RLK), and TIR-NB-LRR genes (TNL), were identified using BLAST2GO 4.1.9 with a

minimum E-value of 1×10^{-3} (Conesa et al. 2005) against the annotated *Arabidopsis thaliana* gene model. This process annotated the function of the genes in the QTL target region using the CDS of genes of the *B. rapa* 'Chiifu'. Genes encoding disease resistance proteins in the targeted region were examined to identify potential candidate genes for each QTL. To keep the comparison consistent in the *CR* gene comparisons, all of the previously identified genes/loci identified using other forms of the reference genome were converted to the latest version (v3.0; Chiifu) and compared with the QTL identified in the current study. The most probable Arabidopsis homolog corresponding to each disease resistance gene, and the class of disease resistance proteins were determined using the CDS of the disease resistance genes in *B. rapa* by Blast search at www.arabidopsis.org. If there was more than one gene, both were taken into consideration.

3.3. Results

3.3.1. Clubroot disease reaction

In the assessment of the reaction to pathotypes 3A, 3D, 3H, and 5X, all of the plants of the R parent ECD02 were resistant (0% DSI) to all four pathotypes, while all plants of the S parent ACDC were highly susceptible (100% DSI). All of the F_1 plants were resistant to all four pathotypes with 0% DSIs (Table 3.1, Figure 3.1). The BC_1S_1 population showed a range of reactions (Figure 3.2). Lines with DSI < 60% were regarded as 'R' and those with DSI ≥ 60% as 'S' (Yu et al. 2017). Based on these criteria, the BC_1 derived from ACDC × (ACDC × ECD02) had a 1:1 (R:S) segregation ratio against each pathotype, based on the DSIs of their BC_1S_1 families (Table 3.1). The 1:1 ratio in BC_1 and resistance reaction of the F_1 plants indicated that a single dominant gene derived from ECD02 controlled the resistance to each pathotype.

The DSI values to the pathotypes in the BC_1S_1 population derived from ACDC × ECD02 were highly correlated (Table 3.2). The strong correlation indicated that the resistance to all four pathotypes (3A, 3D, 3H and 5X) might be controlled by the same or a group of closely linked genes derived from the resistant parent.

As shown in Table S3.1, all plants of the susceptible control DH16516 were also highly susceptible (100% DSI) to each pathotype. NRC11-24 was highly susceptible to pathotypes 3A, 3D, and 5X (100% DSI), but resistant to 3H (0 % DSI). The *B. napus* breeding lines Y12 and Y68 were highly resistant to all four pathotypes (0% DSI).

3.3.2. Alignment with reference genome

DNA short reads from whole-genome sequencing of the parental lines ECD02 and ACDC, and GBS from the 93 BC_1 lines, were aligned with the *B. rapa* reference genome v3.0. The total number of short reads was 214.3 million (M) with 192.1 M sequences aligned (depth coverage 68×), and 96% coverage of the genome for the resistant parent ECD02, and 552.9 M with 478.9 M sequences aligned (depth coverage 169×) and 92% coverage for ACDC (Table S3.2). A total of 432.9 M GBS short reads in the 93 BC_1 lines were obtained, ranging from 1.6 to 9.2 M sequences per line. The mean number of reads aligned with the reference genome from each line was 4.0 M (range 1.4 to 8.5 M, Table S3.2). The coverage ranged from 4.0–8.0% with a mean of 5.7%. (Figure S3.2, Table S3.2).

3.3.3. Identification of SNPs and construction of linkage groups

After sequence assembly, the data were assessed to identify variants and to create a SNP table. There were 93,454 SNPs present in at least 50% of the progeny lines. Of these, 17.9% highly polymorphic markers were selected based on their LOD values; markers with > 15 LOD values were used to create a linkage map. The 16,702 SNPs were further analyzed and tightly

linked markers were selected based on a LOD value of 15–20, which selected 2,539 high-quality markers that were distributed across the 10 chromosomes. The mean number of SNPs on each linkage group was 254, with 306 SNPs on linkage group A10, and 189 SNPs on A07. The mean linkage group length was about 620 cM, and ranged from 530 cM to 820 cM (Figure 3.3).

Among the 2,539 filtered SNPs, 1,195 redundant markers were identified and deleted using QTL IciMapping. The remaining 1,344 high quality, unique SNPs were distributed into 10 chromosomes of A-genome, with 105 to 159 SNPs per chromosome and an average missing rate of 0.27% (Table S3.3). Each SNP was positioned about 3 to 5 cM apart along each chromosome (Table 3.3). The estimated length of the chromosomes ranged from 452 to 757 cM with a mean length of 452 cM. These SNPs were used to identify the association between the clubroot reaction of each line and QTL associated with resistance.

3.3.4. Mapping QTL for resistance to pathotypes 3A, 3D, 3H, and 5X

QTL mapping was performed using 1,344 high quality, polymorphic SNPs in association with the DSI value for each line to each pathotype. A single QTL for resistance to all four pathotypes, designated as *Rcr9*^{ECD02}, was mapped on linkage group A08, with a strong peak associated with SNP markers CF_A08_10575267 and CF_A08_11903476. The SNP marker on the left CI was CF_A08_11903476 (399.5 cM) and on the right CI it was CF_A08_10575267 (402.5). These markers were located in the interval of *Rcr9*, a CR locus identified previously (Yu et al. 2017). However, *Rcr9*^{ECD02} conferred resistance to all four of the pathotypes assessed, whereas *Rcr9* conferred resistance only to 5X. *Rcr9*^{ECD02} was associated with a phenotypic variation explained (PVE) of 68.9 to 77.4% in response to the individual pathotypes, with corresponding LOD values of 24.3 to 31.1 (Table 3.4, Figure 3.4). The Add values of the QTL to resistance were in the range of 64.2 to 74.7 against the pathotypes (Table 3.4, Figure 3.4).

3.3.5. DNA variants and SNP markers in the target region

To confirm the location of *Rcr9*^{ECD02}, the BC_1-BC_1S_1 population was analyzed using conventional linkage mapping. There were 43 R lines and 50 S lines in the BC_1, based on the mean DSIs of four pathotypes in the BC_1S_1 populations. There were 135 high-quality SNP markers identified on chromosome A08, but only two SNP markers (CF_A08_10575267 and CF_ A08_11903476) were closely associated with *Rcr9*^{ECD02}.

To identify more SNP sites in the target region and develop robust SNP markers that could potentially be used for marker-assisted selection, GBS short reads from the R bulk and the S bulk were aligned with the *B. rapa* 'Chiifu' reference genome. There were 5.5 M short reads and 6.8 M assembled into chromosome A08 with template coverage of 14.9% from the R bulk and 15.3% from the S bulk. Further analysis to identify high quality variants uniquely from the R bulk was performed in the target region (10,575,267 to 11,903,476 bp of A08) and an extended region to 12,326,805 bp that included the previously cloned *Crr1* (Suwabe et al. 2003), which was homologous to *BraA08g014480.3C* (12,271,553 to 12,276,276 bp of A08). There were 44 DNA variants consisting of 41 SNPs and 3 InDels in this region (Table S3.4).

A KASP assay was carried out for 22 of the 41 SNPs. Of these 22 SNPs, 14 were polymorphic between the parental lines used for genotyping the 93 BC_1 lines. A linkage map consisting of the 14 SNPs identified with the KASP assay and 2 SNPs identified by QTL analysis was constructed (Figure 3.5 & S3.3). *Rcr9*^{ECD02} co-segregated with CF_A08_11021839, CF_A08_11059924, CF_A08_11466518, CF_A08_11672817, CF_A08_11855997, and CF_A08_11903476, and was flanked by CF_A08_10721706 and CF_A08_12230973, in an interval of 2.2 cM. This interval was upstream of *BraA08g014480.3C* (12,271,553 to 12,276,276 bp of A08), where *Crr1* is located.

3.3.6. Identification of genes encoding disease resistance proteins

Rcr9^{ECD02} was associated with two SNP markers (CF_A08_10721706 and CF_A08_12230973) in the KASP assay. The region between these two markers, which spanned 1.5 Mb on the chromosome, included 219 *B. rapa* genes identified on the reference genome (Table S3.5). Among these 219 genes, there were four potential resistance genes: *BraA08g012910.3C*, *BraA08g012920.3C*, *BraA08g013130.3C* and *BraA08g013630.3C* (Table 3.5).

The first three genes were homologous to the Arabidopsis gene *AT3G05360*, which encodes receptor-like protein 30. The fourth gene was homologous to *AT5G11250*, encoding an atypical TNL protein, *BraA08g014480.3C*, which was homologous to the previously cloned resistance gene *Crr1*. However, this gene was not in the interval previously reported for *Crr1* (Table 3.5).

3.4. Discussion

Clubroot, caused by *P. brassicae*, is an important disease of canola in Canada. All of the initial CR canola cultivars released in Canada have proven to be susceptible to many of the new pathotypes of *P. brassicae* that have emerged in recent years, including 3A, 3D, and 5X identified from CR canola (Strelkov et al. 2018). Pathotype 3A is currently predominant among the new pathotypes, followed by 3D (Strelkov et al. 2018; Hollman et al. 2021).

The *B. rapa* line ECD02 was highly resistant to all four of the pathotypes tested in the current study. All of the F_1 plants were resistant to all four of the pathotypes. Based on the previous observations (Yu et al. 2016; Yu et al. 2017), BC_1S_1 lines with DSI < 60% were likely from resistant BC_1 lines. Therefore, the BC_1S_1 families with DSI < 60% were classified as R and those families with DSI ≥ 60% as S families in this study (Figure 3.3). The BC_1S_1 families

segregated for R:S at a 1:1 ratio. This indicated that resistance was controlled by one dominant gene from the resistant parent ECD02. The DSI values for each line were highly correlated for each of the pathotypes. This indicated that resistance was controlled by a single gene or tightly linked genes. Analysis identified a single QTL, *Rcr9*^{ECD02}, on chromosome A08 conferring resistance to pathotypes 3A, 3D 3H and 5X, which was consistent with the conventional analysis. Both QTL analysis and linkage mapping indicated that *Rcr9*^{ECD02} mapped to the 10,575,267 to 12,331,263 base region of chromosome A08 in *B. rapa* 'Chiifu' v3.0. The name *Rcr9*^{ECD02} was selected because the gene was mapped into the genetic region of *Rcr9* and was originally derived from *B. rapa* line ECD02.

Clubroot severity in the DH lines in response to the individual pathotypes was highly correlated, which indicated that the same or tightly linked genes likely controlled resistance to these pathotypes. However, the identification of QTL in this study was based on relatively coarse gene mapping, so it could not be determined if resistance to the pathotypes was controlled by a single gene or tightly linked genes. Additional studies are in progress. However, strong resistance to Canadian clubroot pathotypes is generally controlled by single dominant genes/loci such as *Rcr1–Rcr9* (Chu et al. 2014; Yu et al. 2016; Huang et al. 2017; Yu et al. 2017; Dakouri et al. 2018; Chang et al. 2019; Huang et al. 2019; and Karim et al. 2020).

Previous studies identified *CRa* on chromosome A03 in ECD02 (Matsumoto et al. 1998; Matsumoto et al. 2005; Ueno et al. 2012; Fredua-Agyeman et al. 2020). However, *CRa* was not found in the current mapping population. In nature, *B. rapa* is self-incompatible and requires cross-pollination. As a result, it is unlikely that ECD02 is a homogenous line. It is therefore possible that the mapping population derived from the donor plant used for this study did not carry *CRa*.

A previous study has identified three QTL in *B. rapa* line T19 that conferred resistance to several pathotypes (Yu et al. 2017). T19 had originated from the German turnip 'Pluto'. A single QTL on chromosome A03, designated *Rcr4*, conferred resistance to pathotypes 2F, 3H, 5I, 6M and 8N (Yu et al. 2017), but was not effective against 3A, 3D or 5X (Table S3.1). Although *Rcr8* on chromosome A02 and *Rcr9* on A09 also conferred resistance to pathotype 5X (Yu et al. 2017), they did not confer resistance to 3A, 3D or 3H (Table S3.1). Therefore, $Rcr9^{ECD02}$ from ECD02 is likely different from the three CR loci from T19.

Previously, *Rcr9* for resistance to pathotype 5X had been identified in the *B. rapa* breeding line T19 (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*, $Rcr9^{wa}$ (Karim et al. 2020), $Rcr9^{ECD01}$ (Yu et al. 2021) and a QTL identified in Rutabaga (Hasan & Rahman 2016). However, the BC_1S_1 families that carried *Rcr9* were resistant to 5X but not to 3A, 3D or 3H (Table S3.1). This difference in phenotype indicated that *Rcr9* differed from $Rcr9^{ECD02}$. In addition, another resistance locus, designated as $Rcr9^{wa}$, has been identified from a differential line in the ECD set. It originated from the turnip 'Waaslander' (ECD04), was mapped to the same interval as *Rcr9* and provided resistance to pathotype 5X (Karim et al. 2020). Based on flanking markers, $Rcr9^{wa}$ was mapped to the 12.3–12.6 Mb region of chromosome A08 (a smaller interval than *Rcr9*), which is distinct and separate from $Rcr9^{ECD02}$. Similarly, another resistance QTL/gene that originated from 'Waaslander' and conferred resistance to pathotype 3H, designated *Rcr3*, was mapped to chromosome A08 and was flanked by SNP markers on the 11.3–11.6 Mb region in the *B. rapa* 'Chiifu' reference genome v3.0 (Karim et al. 2020). The position of *Rcr3* was in the interval of $Rcr9^{ECD02}$. However, it conferred resistance to pathotype 3H, but not to 5X (Karim et al. 2020). Moreover, the QTL identified in the Rutabaga was resistant to pathotype 2, 3, 5, 6, and

8 (Hasan & Rahman 2016). There were no information on resistant reaction to the new clubroot resistance-breaking pathotypes. Therefore, it was not possible compare the identified QTL position with currently identified *Rcr9*^{ECD02}. Finally, there has been a QTL identified from ECD01, designated as *Rcr9*^{ECD01}, which conferred resistance to 3D, 3H and 5X, but resistance to 3A was only apparent when resistance alleles were present at both loci *Rcr9*^{ECD01} and *Rcr10*^{ECD01} (Yu et al. 2021). It was mapped to the 12.0 to 14.5 Mb region of A08 in *B. rapa* 'Chiifu' v3.0, partially overlapping with *Rcr9*^{ECD02} (Figure 3.11).

In addition, the gene *BraA.CR.b* for resistance to pathotype 3H was previously identified from the turnip differentials ECD01, ECD02, ECD03 and ECD04 and mapped to 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. Similarly, several genes/loci 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 to 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 not located in the *Rcr9*^{ECD02} genomic region. In addition, breeding lines carrying *Crr1* were not resistant to the pathotypes of *P. brassica* assessed in this study (unpublished data). Therefore, *Rcr9*^{ECD02} is unlikely the same as *Crr1*. The relationship between *Rcr9*^{ECD02} and *CRs* (Laila et al. 2019), *PbBa8.1* (Chen et al. 2013) and *qBrCR38-2* needs to be determined.

The *CR* genes *CRa*, *CR*^{kato} and *Crr1* in *B. rapa* have been cloned. They all encoded TNL class disease resistance proteins (Ueno et al. 2012; Hatakeyama et al. 2013; Hatakeyama et al. 2017). There were four genes annotated as disease resistance proteins in the *Rcr9*^{ECD02} interval. One of them (*BraA08g013630.3C*) belongs to the TNL class, while the other three

(*BraA08g012910.3C*, *BraA08g012920.3C* and *BraA08g013130.3C*) encode receptor-like protein 30. Further studies are required to identify which of these genes is the main gene responsible for clubroot resistance, and to determine whether all four genes are necessary for resistance to the four pathotypes. This can be addressed when the gene is cloned.

3.5. Conclusion

In the current study, a single co-localized QTL *Rcr9*^{ECD02} was identified on chromosome A08 of the *B. rapa* line ECD02 using 93 BC_1S_1 lines; this QTL conferred resistance to *P. brassicae* pathotypes 3A, 3D, 3H, and 5X. A set of high quality markers consisting of 1,344 SNPs distributed across the 10 chromosomes was developed and used for QTL mapping. The identified QTL *Rcr9*^{ECD02} explained 68.9-74.4% of the phenotypic variation with LOD values of 24.3 to 31.1. Bulked segregant analysis and KASP genotyping were also used to identify 14 robust SNP markers linked to *Rcr9*^{ECD02}, with six markers completely associated with the QTL, and four markers flanking the loci in an interval of 2.2 cM in the population. Four TNL genes encoding disease resistance proteins were found in the QTL region. The QTL *Rcr9*^{ECD02} represents a valuable genetic resource for the development of new CR canola cultivars. The availability of multiple resistance genes can provide opportunities for the rotation of sources of resistance, thereby contributing to resistance stewardship and more sustainable management of clubroot of canola.

3.6. Tables

Table 3. 1. Correlation matrix for clubroot severity (disease severity index, DSI) in BC_1S_1 lines derived from ACDC × (ACDC × ECD02) inoculated with four pathotypes (3A, 3D, 3H, and 5X) of *Plasmodiophora brassicae* under controlled conditions.

Pathotypes	3A	3D	3H	5X
3A	1.00			
3D	0.93**	1.00		
3H	0.95**	0.92**	1.00	
5X	0.89**	0.91**	0.86**	1.00

** significant at $P \leq 0.01$.

Table 3. 2**.** Marker distribution throughout the *Brassica rapa* parent genotypes ACDC and ECD02, and BC_1 population derived from ACDC × (ACDC × ECD02).

Materials		Total # of sequence ($\times 10^5$)	Assembled sequence ($\times 10^5$)	Fold coverage	# of bases / sequence ($\times 10^3$)	Total # of SNPs ($\times 10^5$)
Parental	ACDC	5529	4789	0.92	86.7	62
lines	ECD02	2143	1921	0.96	89.6	78
BC1	Mean	44.6	40.3	0.006	0.09 ±	2.38 ±
	± SE	± 1.75	± 1.56	± 0.0003	0.004	0.043
	Total	4329	3911	0.57	90.3	231

Table 3. 3**.** Distribution of SNPs on linkage groups of a BC_1S_1 population of *Brassica rapa* [ACDC × (ACDC × ECD02)] used to identify QTL for resistance to pathotypes 3A, 3D, 3H, and 5X of *Plasmodiophora brassicae*.

Linkage group	Number of Markers	Total Length (cM)	Mean Length between Adjacent Markers(cM)
A01	136	515.4	3.79
A02	130	756.6	5.82
A03	159	547.0	3.44
A04	115	630.1	5.48
A05	146	648.2	4.44
A06	141	693.3	4.92
A07	131	743.8	5.68
A08	135	561.8	4.16
A09	105	451.9	4.30
A10	146	475.7	3.26
Total	1344	6023.8	-
Maximum	159	756.6	5.82
Minimum	105	451.9	3.26
Mean	134	602.4	4.53

Table 3. 4**.** Additive QTL effect on clubroot severity of a *Brassica rapa* BC_1S_1 population, derived from ACDC × (ACDC × ECD02), after inoculation individually with four pathotypes (3A, 3D, 3H, 5X) of *Plasmodiophora brassicae* and corresponding markers linked to the QTL '*Rcr9*^{ECD02}' on chromosome A08.

Pathotype	Interval	Peak				Flanking markers		Nearest SNP to peak	
	(cM)	LOD	Position	PVE (%)	Addit.* (%)	Left marker	Right marker	Name	position
3A	400.5–402.5	30.1	401	73.8	72.7	A08_11903476	A08_10575267	A08_11903476	400.5
3D	400.5–402.5	31.1	401	76.6	74.7	A08_11903476	A08_10575267	A08_11903476	400.5
3H	400.5–402.5	28.0	401	73.1	74.0	A08_11903476	A08_10575267	A08_11903476	400.5
5X	400.5–402.5	24.3	401	68.1	64.2	A08_11903476	A08_10575267	A08_11903476	400.5

*Alleles from ECD02

Table 3. 5**.** Identified major genes near QTL '*Rcr9*^{ECD02}', on chromosome A08, and their characteristics with functions.

Gene name	*B. rapa* gene location (base)	Length (base)	Gene description from Blas2Go	Gene functions from Blast2Go
BraA08g012910.3C	11232393….11235117	2724	Disease resistance	Defense response to fungus- oomycetes, signal transduction, detection of a molecule of fungal origin.
BraA08g012920.3C	11248087….11250727	2640	Disease resistance	Defense response to fungus- oomycetes, signal transduction, detection of a molecule of fungal origin.
BraA08g013130.3C	11388577….11391460	2883	Disease resistance	Defense response to fungus, signaling pathway, kinase activity
BraA08g013630.3C	11696216….11696785	569	Disease resistance (TIR-NBS-LRR class) family	Signal transduction, regulation of cellular response to stress, ADP binding, and defense response.

3.7. Figures

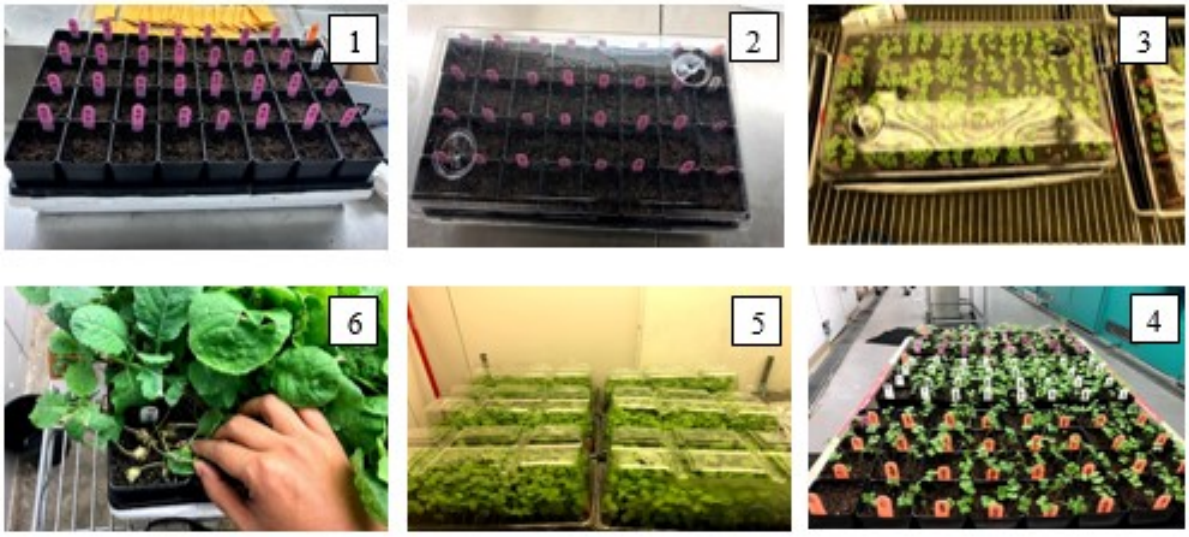


Figure 3. 1**.** Steps in testing a BC_1S_1 population for reaction to four pathotypes of *Plasmodiophora brassicae* under controlled conditions. Panels 1–6 illustrate the sequential steps followed in the protocol.

Figure 3. 2**.** Clubroot disease severity rating scale, where: 0 = no galls on roots, 1 = a few small galls, 2 = moderate galls, and 3 = severe galls.

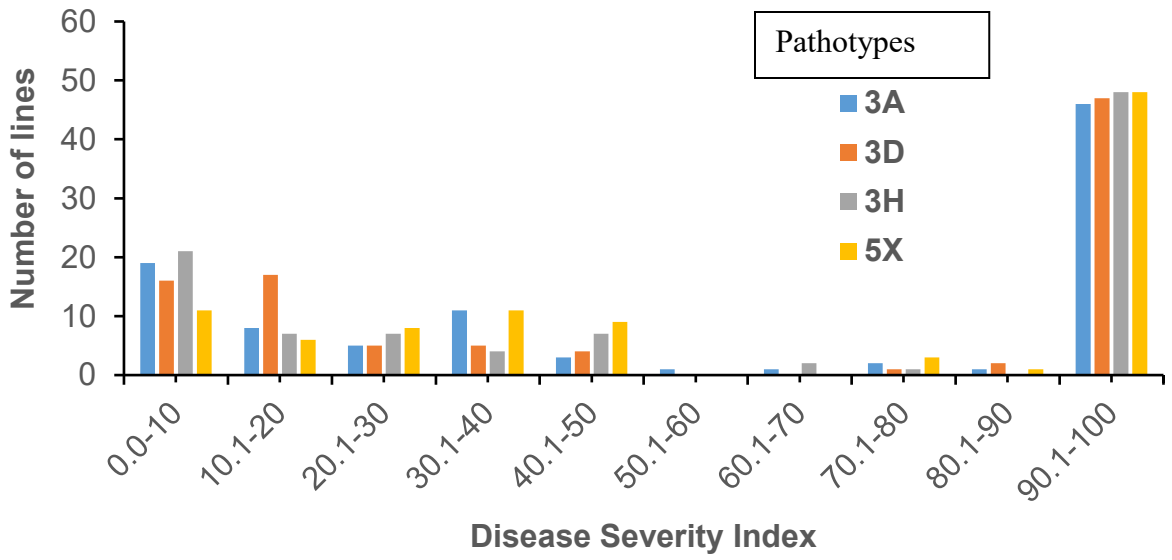


Figure 3. 3**.** Clubroot severity (disease severity index, DSI) of 93 lines of a BC_1S_1 population derived from ACDC × (ACDC × ECD02) and inoculated with pathotypes 3A, 3D, 3H and 5X of *Plasmodiophora brassicae* in a growth chamber.

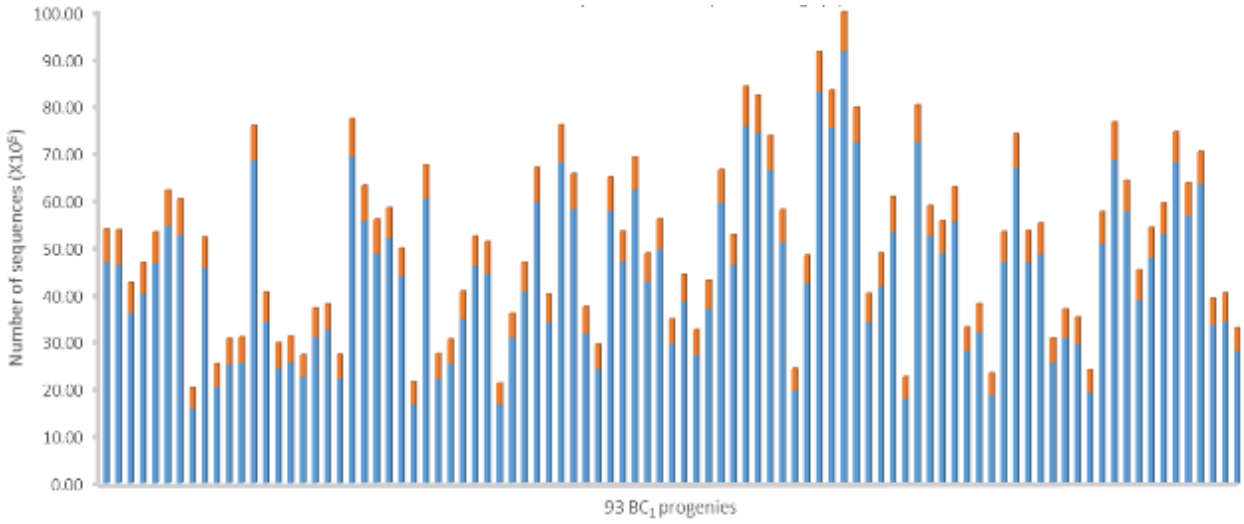


Figure 3. 4**.** The proportion of SNPs within the sequence of 93 BC_1 lines (orange bars are sequences with SNPs, blue bars are sequences without SNPs).

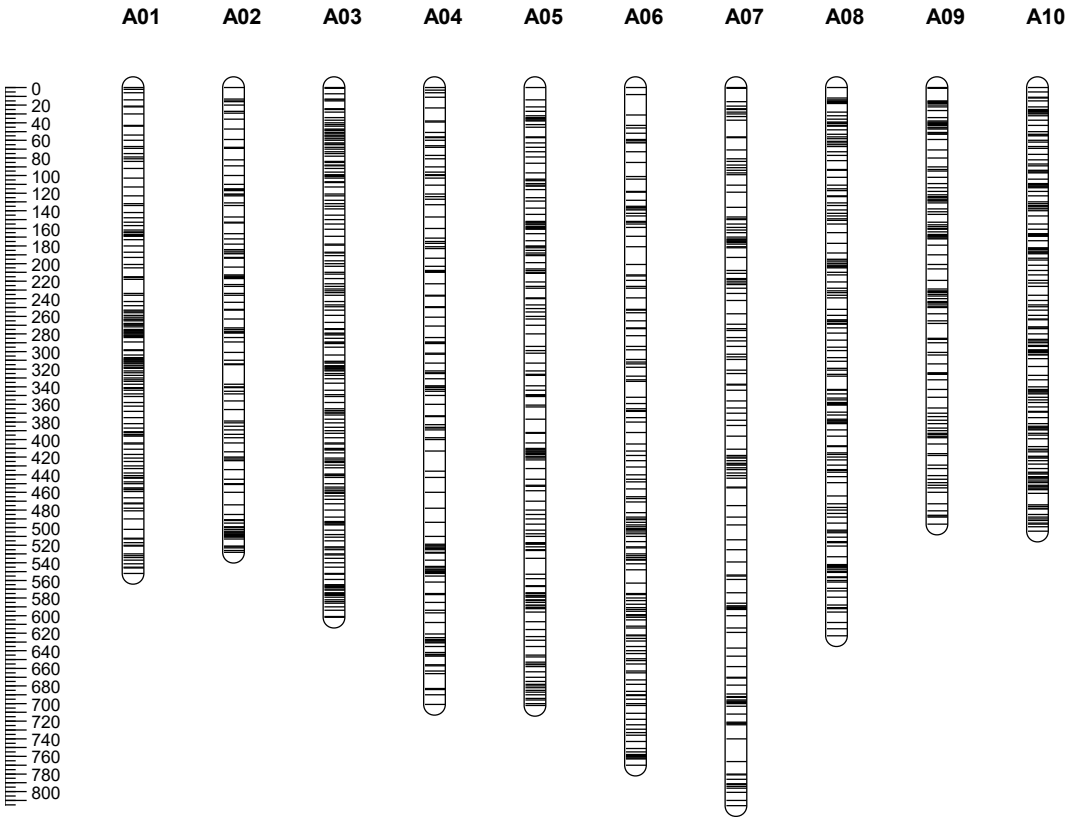


Figure 3. 5**.** Linkage map of the sequences of a BC_1S_1 population derived from ACDC × (ACDC × ECD02) consisting of 2,539 SNPs. The vertical scale on the left indicates the genetic distance in centimorgans (cM).

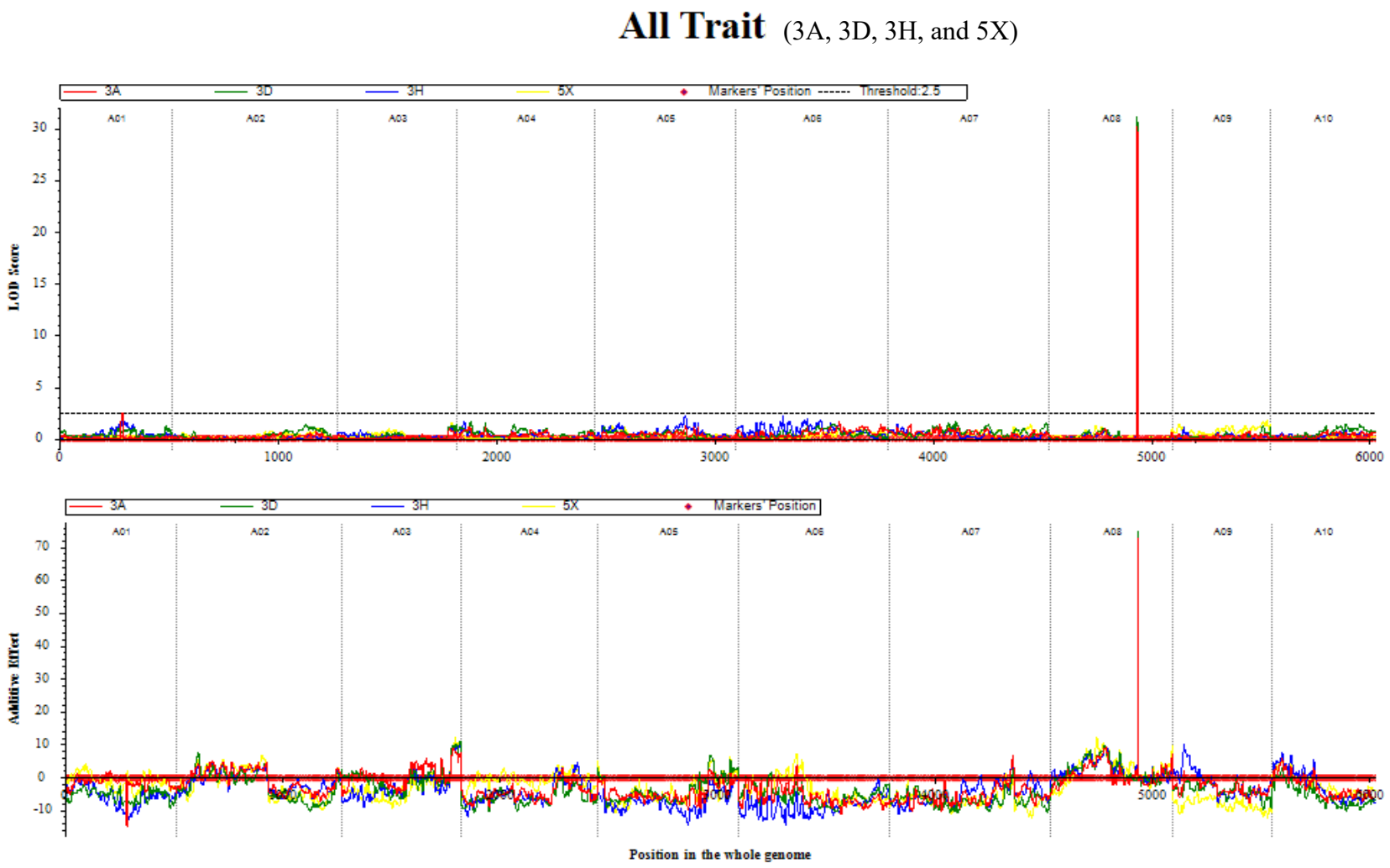


Figure 3. 6**.** LOD plot and the additive effect of clubroot resistance QTL against pathotypes 3A, 3D, 3H, and 5X of *Plasmodiophora brassicae* with a linkage group of a BC_1S_1 population derived from ACDC × (ACDC × ECD02).

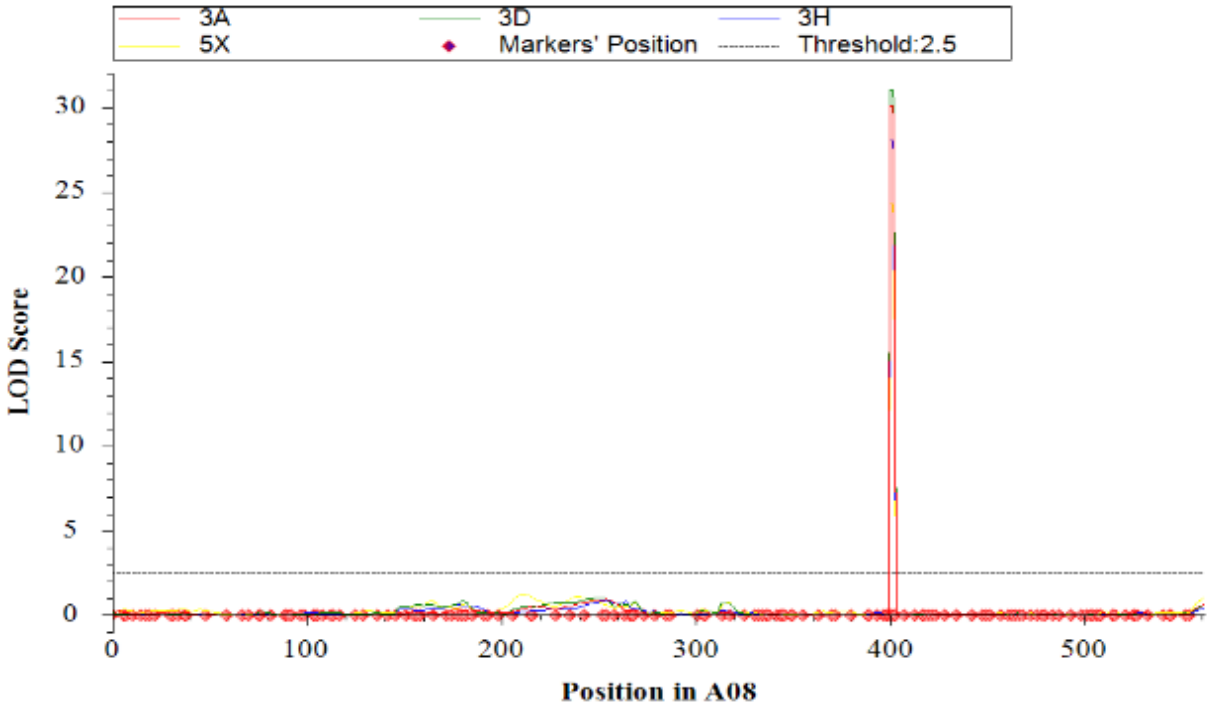


Figure 3. 7**.** The peak in the LOD score indicates the QTL position and additive effect of QTL on chromosome A08 of *Brassica rapa* cultivar ECD02 that provide the resistance reaction to the four pathotypes (3A, 3D, 3H, and 5X) of *Plasmodiophora brassicae*.

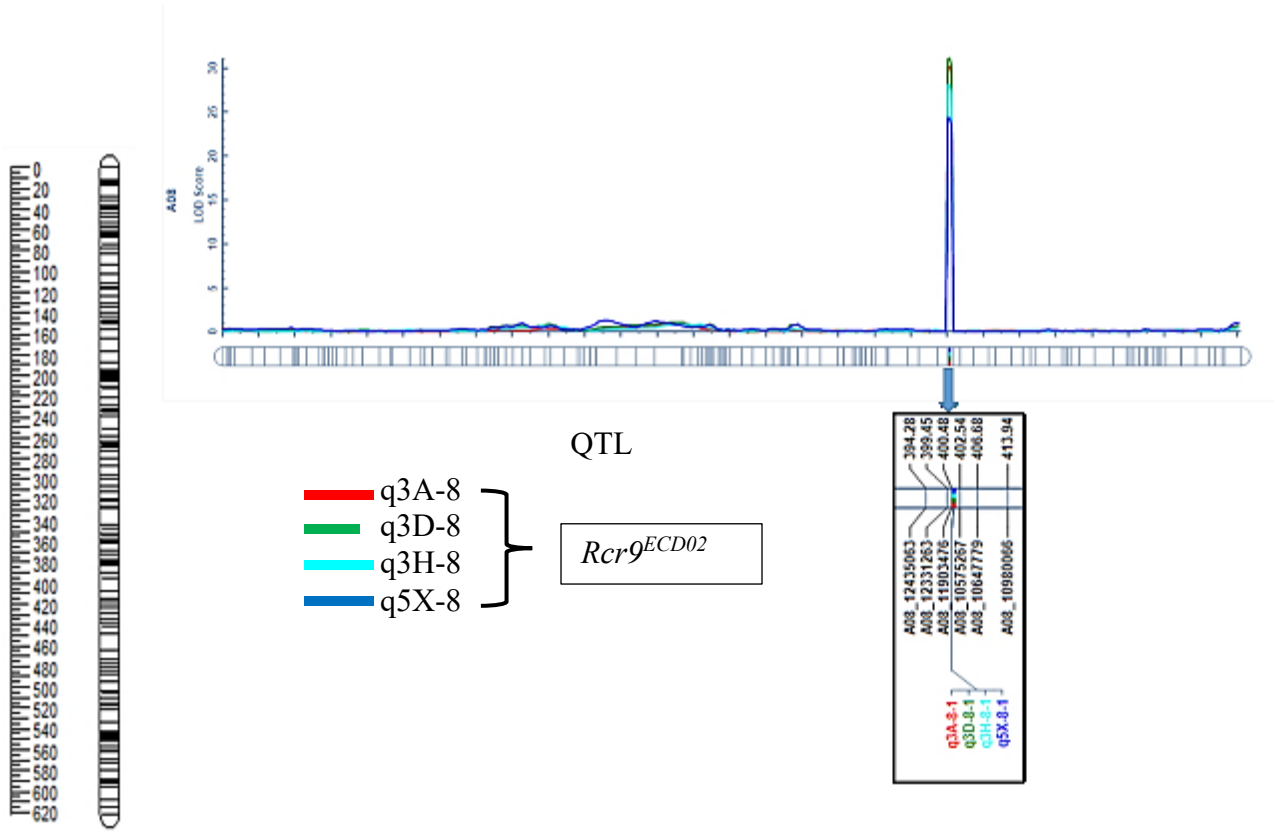


Figure 3. 8**.** The QTL likelihood profile of four QTL for clubroot resistance, the genetic position intervals between those QTL, and physical positions of the marker on chromosome A08 of *Brassica rapa* line ECD02. The vertical scale on the left indicates the genetic distance in centimorgans (cM).

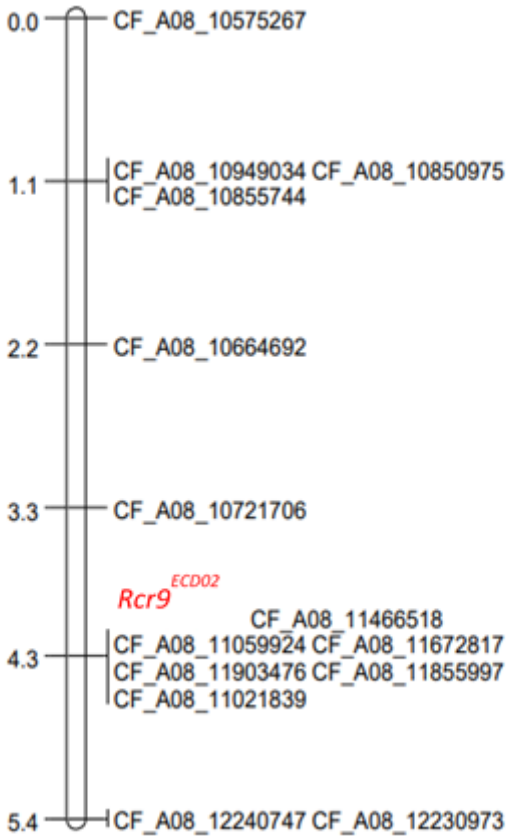
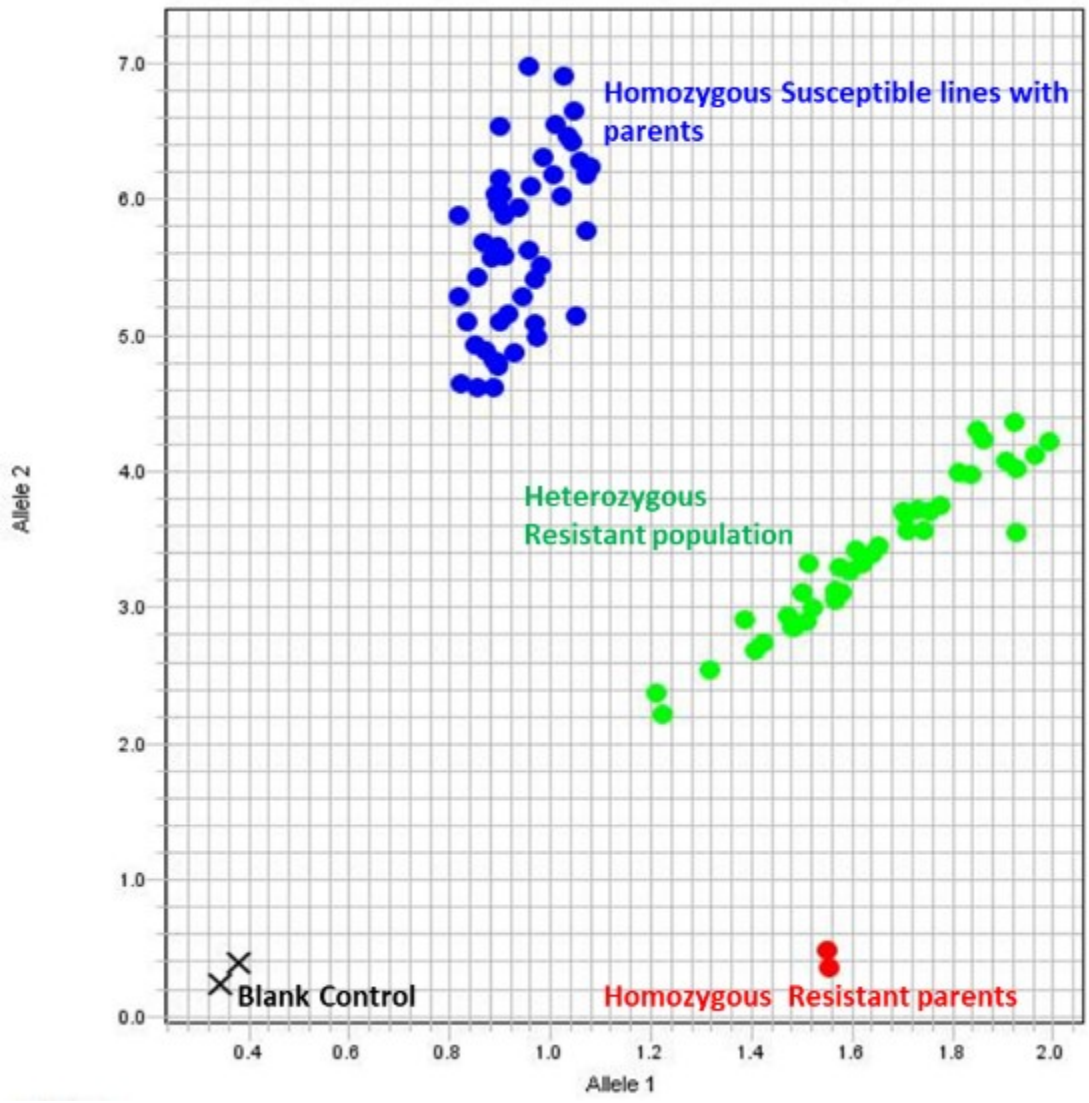


Figure 3. 9**.** Linkage map of A08 based on 14 SNPs from a KASP assay and 2 SNPs identified by QTL analysis, illustrating the relative position of *Rcr9*^{ECD02} on this chromosome.



an

Figure 3.10**.** Allelic discrimination plots of KASP SNP marker analysis of *Brassica rapa* BC_1S_1 populations, derived from ACDC × (ACDC × ECD02), where ACDC is the susceptible parent, and ECD02 is the resistant parent of the population.

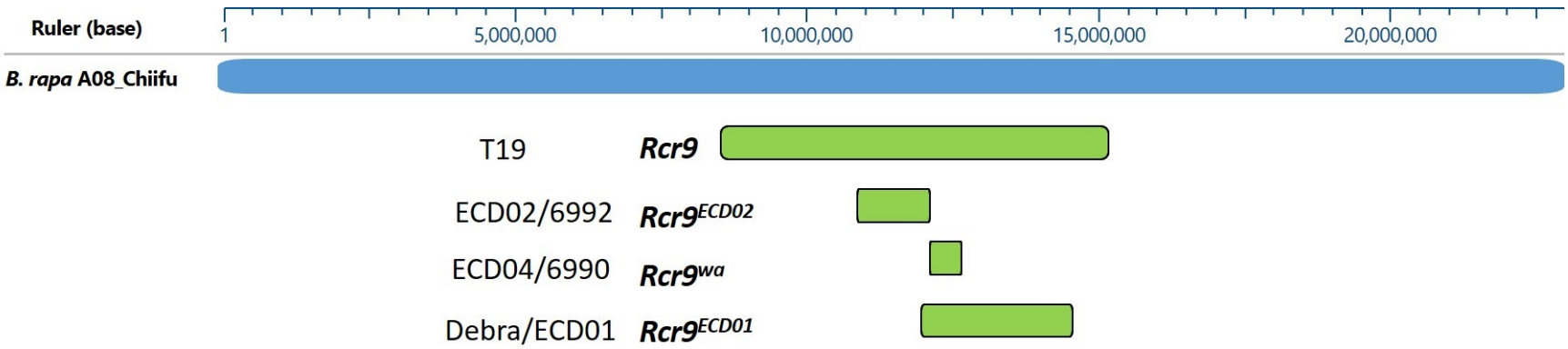


Figure 3.11. Location of the identified QTL *Rcr9*^{ECD02} on A08 chromosome *Brassica rapa* genome v3.0 (Chiifu) in relation to the previously identified QTL *Rcr9* (Yu et al. 2017), *Rcr9*^{wa} (Karim et al. 2020) and *Rcr9*^{ECD01} (Yu et al. 2021) from different populations.

Chapter 4. Genotyping-by-sequencing reveals a major QTL for resistance to four virulent pathotypes of *Plasmodiophora brassicae* in the winter oilseed rape (*Brassica napus*) 'AAFC695'

4.1. Introduction

Oilseed rape or canola (*Brassica napus* L.) is one of the most important edible oil crops worldwide. In Canada, canola contributes $29.9 billion annually to the national economy (Canola Council of Canada 2020). Unfortunately, clubroot disease, caused by the obligate parasite *Plasmodiophora brassicae* Wor., represents a significant threat to the sustainable production of this and other cruciferous crops (Dixon 2009). In western Canada, clubroot has been spreading through the main canola-growing regions of Alberta, Saskatchewan and Manitoba since the 2000s (Strelkov & Hwang 2014); the disease is most severe in Alberta, where clubroot had been confirmed in over 3300 fields by 2020 (Strelkov et al. 2021). Infection by *P. brassicae* results in the formation of large galls on the roots of susceptible hosts, which interfere with water and nutrient uptake from the soil. Under conditions favorable for disease development, yield losses on canola may approach 100% (Pageau et al. 2006; Botero-Ramírez et al. 2022). The management of clubroot can be difficult, since *P. brassicae* produces very large numbers of resilient resting spores that persist in the soil for many years (Braselton 1995; Wallenhammar 1996), serving as inoculum for future infections.

While a variety of strategies have been evaluated for the control of clubroot in canola, including, among others, liming of the soil to increase its pH (Hwang et al. 2014; Fox et al. 2022) and planting of bait crops to deplete soil resting spore concentrations (Ahmed et al. 2011), the deployment of genetically resistant cultivars is the most effective and economical

management approach (Peng et al. 2014; Rahman et al. 2014). Clubroot-resistant (CR) canola was first introduced to Canada in 2009, and quickly became the most widely used tool to manage the disease (Strelkov & Hwang 2014). The genetic basis for this resistance, while not in the public domain, appeared to be similar in most cultivars and was derived from the European winter oilseed rape cultivar 'Mendel' (Fredua-Agyeman et al. 2018). Unfortunately, the virulence of *P. brassicae* populations can shift quickly in response to the selection pressure exerted by resistant hosts (LeBoldus et al. 2012), and 'novel' pathotypes of *P. brassicae* able to overcome host resistance were detected in Alberta by 2013 (Strelkov et al. 2016). The distribution and number of new pathotypes has been increasing yearly, with approximately 17 resistance-breaking pathotypes, as defined on the Canadian Clubroot Differential (CCD) set, reported to date (Strelkov et al. 2018; Strelkov et al. 2021; Hollman et al. 2021). These include pathotypes 3A (predominant resistance-breaking pathotype), 2B (widest host range), 3D (second most-common after 3A) and 5X (first resistance-breaking pathotype identified in Canada).

Clubroot resistance in *B. napus* (AACC, 2n = 38) is primarily descended from the A-genome of *B. rapa* (Diederichsen et al. 2006; Peng et al. 2014). *Brassica rapa* (AA), especially turnip (*B. rapa* ssp. *rapifera*), possesses multiple independent and race or pathotype-specific *CR* genes in its genome profile (Crute et al. 1980; Toxopeus and Janssen 1975). Indeed, to date, more than 18 CR loci/genes have been identified and mapped in *B. rapa*: *CRa, CRb, CRd, CRk Crr3, PbBa3.1, PbBa3.3, Rcr1, Rcr2, Rcr4, Rcr5, Crr1, CRs, Rcr9, Rcr3, Rcr9^{wa}, Rcr9^{ECD01}, CRc, Rcr8, Crr2,* and *Crr4* (Chen et al. 2013; Chu et al. 2014; Hirai et al. 2004; Huang et al. 2019; Huang et al. 2017; Karim et al. 2020; Laila et al. 2019; Matsumoto et al. 1998; Pang et al. 2018; Piao et al. 2004; Saito et al. 2006; Sakamoto et al. 2008; Suwabe et al. 2003; Suwabe et al. 2006; Yu et al. 2017; 2021), with most of these genes found in European fodder turnip

(Sakamoto et al. 2008; Yu et al. 2017; Karim et al. 2020; Diederichsen et al. 2009a; Piao et al. 2009). Based on several *B. rapa* gene mapping studies, chromosome A03 is a hotspot for most of the *CR* genes, followed by chromosomes A08, A02, A01, and A06.

In contrast to the major resistance genes on the A-genome, the C-genome of *B. napus* includes many QTL for clubroot resistance (Piao et al. 2009; Rahman et al. 2014; Dakouri et al. 2018). Around 37 resistance QTL and major genes have been identified and mapped in the C-genome, namely *CR2a, CR2, Pb-Bo1, Pb-Bo2, Pb-Bo3, -o4, Pb-Bo5a, Pb-Bo5b, Pb-Bo8, Pb-Bo9a, Pb-Bo9b, PbBo1, Pb-Bo (Anju) 1, Pb-Bo (Anju)2, Pb-Bo (Anju)3, Pb-Bo (Anju)4, pb-3, pb-4, QTL1, QTL3, QTL9, CRQTL-YC, CRQTL-GN_1, CRQTL-GN_2, DIC.I-1, DIC.II-1, Rcr7, PbC4.1, PbC6, PbC7.1, PbC7.2, PbC8, PbC9.1, PbC3, PbC4.2, PbC7.3,* and *PbC9.*2 (Farid et al. 2020; Figdore et al. 1993; Grandclément and Thomas 1996; Landry et al. 1992; Peng et al. 2018; Strelkov et al. 2018; Voorrips and Kanne 1997). As resistance mediated by *CR* genes plays an important role in protecting *Brassica* crops from clubroot, the identification and mapping of these genes is important for the development of clubroot-resistant canola varieties (Ce et al. 2021).

High throughput Next-Generation Sequencing (NGS) has enabled a new era in the genome sequencing of many plant species, including *Arabidopsis thaliana* L. Heynh. (Schranz and Mitchell-Olds 2006) and polyploid *Brassica* species such as *B. napus* (Rousseau-Gueutin et al. 2020). Next-Generation Sequencing, especially genotyping-by-sequencing (GBS), is a cheap and highly efficient technology to map specific gene loci (Mammadov et al. 2012; Wang et al. 2020; Rajendran et al. 2022) and identify candidate genes linked to the mapped loci. This technology has been widely used to identify single nucleotide polymorphisms (SNPs) (Elshire et al. 2011; Song et al. 2020; Wang et al. 2020), construct reference genomes and haplotype maps,

evaluate genetic diversity and conduct association studies of many important crops including canola (Wang et al. 2011; Yu et al. 2017; Dakouri et al. 2021; Mundada et al. 2022).

Given the recent emergence of new pathotypes of *P. brassicae* able to overcome host resistance, gene mapping to identify novel resistance sources and incorporate them into breeding programs is important for the effective management of clubroot. While the development of clubroot-resistant canola can be achieved via the introgression of resistance from allied species, the introduction of resistance from the same species is an easier approach. Dakouri et al. (2021) identified various *B. napus* accessions with good resistance to several new pathotypes of *P. brassicae*. One of these accessions, AAFC695 (corresponding to the winter oilseed rape cv. 'AAFC695' from France; https://cgngenis.wur.nl), was resistant to a collection of 12 *P. brassicae* pathotypes, including pathotypes 3A, 2B and 3D (Chapter 2) and 5X (Dakouri et al. 2021). The present study was undertaken to identify and map the *CR* genes in this host via the use of high throughput NGS technology, and to develop molecular markers to accelerate canola breeding programs for the development of CR cultivars. Moreover, the identified and mapped genes could also be used to pyramid resistance genes within the same host genotypes, thereby increasing the durability of resistance against newly emerging pathotypes of *P. brassicae*.

4.2. Methods

4.2.1 Parent selection and F_1 development

Brassica napus accession AAFC695, the winter oilseed rape cv. 'AAFC695', was obtained from the Centre for Genetic Resources, the Netherlands (https://www.wur.nl/en/Research-Results/Statutory-research-tasks/Centre-for-Genetic-Resources-the-Netherlands-1.htm). It is resistant to many *P. brassicae* pathotypes, including pathotypes 3A, 2B, 3D, and 5X, which are able to overcome the resistance found in many

Canadian canola cultivars (Chapter 2; Dakouri et al. 2021). Given its nature as a winter type, 'AAFC695' plants were vernalized for 6 weeks at 4°C. Following vernalization, some of the plants were selected to make a cross with a spring type, the doubled haploid (DH) *B. napus* line DH16516 (a universally clubroot-susceptible DH line originating from the *B. napus* 'Topas'), to produce the F_1 hybrids.

4.2.2. Development of a DH population from F_1 plants

Four resistant F_1 plants from the cross above were selected for microspore culture to develop the DH population. The plants were transferred to 15 cm-diam. pots and allowed to grow under controlled conditions (21°C ± 2°C with a 16 h photoperiod) for seven weeks, followed by a vernalization period at 4°C for six-weeks. After vernalization, the plants were transferred to a growth chamber maintained at 10°C with a 14 h/10 h day/night cycle. The development of the DH population was carried out following Coventry et al. (1988) with some modifications. At bud formation, the buds were collected in Falcon tubes, and buds 3.5 to 4.5 mm in length were selected under a dissecting microscope and washed with distilled water in a 100 mL beaker. After washing, the buds were soaked in 3% bleach (sodium hypochlorite) for 10 min and then rinsed three times with sterile Milli-Q Millipore water. The buds were subsequently placed in a 50 mL glass beaker and homogenized in 25 mL liquid Gamborg B5 medium (Gamborg et al. 1968) with a sterile glass rod. The homogenized bud suspension was passed through two-layers of nested sterile filters [(64 µm top (NTX64) and 41 µm bottom (NTX41)] into a sterile Falcon tube. The Falcon tube was centrifuged three times at 130g for 3 min each time, with the supernant decanted and the pellet washed with B5 liquid washing medium in between centrifugations. Following a final wash, the microspore pellet was resuspended in a 25 mL NLN 13 (pH 6.0-6.2) medium (Lichter, 1982) and poured into individual Petri dishes, with 5

ml of microspore suspension per dish. The Petri dishes were sealed with parafilm and incubated under darkness in an incubator at 32°C for 72 h to provide a heat shock treatment. They were then transferred to an incubator maintained at 30°C for 14 days. Following this incubation period, the Petri dishes were placed in a cardboard box, sealed to ensure darkness inside, and placed on a rotary shaker at 60 rpm for 3 days. They were then moved under light and kept under aerobic conditions for the next 4-7 days to allow the growth of the embryos. Once the embryos developed into a torpedo shape, the Petri dishes were removed from the shaker and placed in a refrigerator at 4°C for 2-7 dyas. The well-developed embryos were then transferred to solid B5 medium in Petri dishes and placed at room temperature under a 16/8 h day/night regime, so the embryos could develop into plantlets.

Plantlets with well-developed roots and true leaves were transferred to a soilless growth medium (Sunshine Mix 3, TerraLink Horticulture Inc., Abbotsford, BC) and covered with transparent cups for about a week. At that point, the bigger plantlets were transferred to larger pots and were grown under standard conditions (21°C ± 2°C, 16 h photoperiod) in a greenhouse for 6 weeks.

Some DH plants did not initiate bud production until 6 weeks after planting, so those plants were vernalized in a growth chamber at 4°C and16 hr light / 8 hr dark for 8 weeks. All of the plants were individually covered with a plastic pollination bag to prevent outcrossing and to produce a DH population. Plants with visible pollen production were regarded as DH plants, and seeds from one plant were harvested and stored as a DH line. The plants with smaller-sized flowers or petals and lacking pollen grains were considered haploid individuals and were discarded from the population. One hundred and two DH lines were obtained from the F_1 donor plants of the cross between ‘AAFC695’ and DH16516.

4.2.3. Inoculum preparation and phenotyping plants for resistance to clubroot

The parents 'AAFC695' and DH16516, the F_1 plants, and the 102 DH lines were tested for their reaction to each of the *P. brassicae* pathotypes 3A (field isolate F.3-14), 3D (F.1-14), 3H (P.41-14), and 5X (field isolate LG02). The DH lines AAFC-Y12 and AAFC-Y68, carrying the *CR* QTL/genes $Rcr9^{ECD01}$ and $Rcr10^{ECD01}$ and resistant to pathotypes 3A, 3D, 2B and 5X (Yu et al. 2021), were included as resistant controls; the DH line NRC11-24 (Nutrien Ag Solutions, Saskatoon, SK) and a Canadian canola cultivar '45H29' (Pioneer Hi-Bred, Caledon, ON), resistant to pathotype 3H but susceptible to pathotypes 3A, 3D and 5X, were included as susceptible checks for the latter three pathotypes. The pathotypes were maintained as frozen root galls on NRC11-24 (3A, 3D, and 5X) or DH16516 (3H).

Inoculum was prepared following Karim et al. (2020). Briefly, about 100 g of frozen galls infected with each of the pathotypes was soaked in 200 mL distilled water in a beaker for 30-60 min. The thawed galls were then cut into small pieces while still under water, and homogenized in a stainless steel blender for three mins. The resulting homogenate was strained through two-layers of fine mesh nylon cloth, and the *P. brassicae* resting spore concentration was estimated with a hemocytometer. The spore concentration was adjusted to 1×10^7 resting spores mL^{-1} solution with sterile distilled water as needed (Strelkov et al. 2006), and the inoculum was stored in at -20°C until use.

Seedlings were grown in 10 cm × 10 cm cells in 32 cell trays filled with Sunshine Mix 3 potting medium (TerraLink Horticulture Inc.). The potting medium was saturated with water, any excess water was drained off, and 6-8 seeds were sown in each cell to obtain at least six seedlings per cell. The seeds were then covered by pressing the potting mix around each seed, and the trays was covered with a transparent lid and moved to a greenhouse maintained at 21°C

± 2°C with a 16 h photoperiod. Seven days after seeding, each cell received 15 ml of *P. brassicae* inoculum, prepared as described above, with the volume of the spore suspension selected to ensure that it was sufficient to soak the roots of all of the seedlings. The seedlings were then grown in the greenhouse for another six-weeks, with watering as required. At this point, the plants were uprooted and evaluated for clubroot severity on a 0-3 scale based on Kuginuki et al. (1999), where: 0 = no galls, 1 = a few small galls, 2 = moderate galling, and 3 = severe galling of the roots. The individual clubroot severity ratings were used to calculate a Disease Severity Index (DSI, 0-100%) for each treatment following Horiuchi and Hori (1980) as modified by Strelkov et al. (2006).

A host genotype was regarded as resistant (R) if the DSI was ≤ 30% or as susceptible (S) if the DSI > 30%. Six seedlings per host genotype were tested with each pathotype in the first round of screening. In most cases, the clubroot reaction of the tested lines was clear, with seedlings showing similar and consistent disease development. However, for some treatments, additional testing was required to confirm the host reaction. In these cases, the genotypes were tested an additional three times with each pathotype, following the same procedure described above. Only those lines showing consistent results across all three repetitions of the experiment were included in further analyses, with the highest DSI obtained taken as the reaction of the validated line(s) to the tested pathotypes to run the mapping work.

4.2.4. Basic statistics and correlation study

Statistical analysis of the phenotypic data was run on the QTL IciMapping platform (Meng et al. 2015) to confirm its quality for QTL analysis. Correlation analysis of reactions to pathotypes 3A, 3D, 3H, and 5X was performed with Microsoft excel. A Chi-square (χ^2) test for

goodness of fit was carried out to calculate the inheritance pattern of the resistance to the tested pathotypes.

4.2.5. Genotyping-by-sequencing

Genomic DNA from 3-4 week-old seedlings of the 102 DH lines and the two parental lines was extracted with a DNeasy Plant Mini Kit (Qiagen) as per the manufacturer's instructions. The quality of the extracted DNA was evaluated on a Nanodrop2000 (Thermo Scientific), Qubit Fluorometer, and Microplate Reader and sample integrity was confirmed by spectrophotometer and by agarose gel electrophoresis. A DNA library was constructed following the protocol of Dakouri et al. (2018). The complexity of the extracted DNA was reduced by digesting the DNA with a methylation-sensitive restriction enzyme ApeKI. The 100 bp fragments produced from the enzymatic digestion were ligated to enzyme-specific adapters and amplified using PCR. Following library construction, the high-quality DNA from the 102 lines and the parent genotype 'AAFC695' was sequenced on an Illumina platform with pair-end sequencing at BGI Americas Corp (Cambridge, MA, USA). Whole genome sequencing of the parent line DH16516 was performed at the Plant Biotechnology Centre (Saskatoon, SK, Canada). The obtained sequences were aligned on the SeqMan NGen 16 (DNASTAR, Madison, WI, USA) platform with the *B. napus* reference genome ZS11 (Song et al. 2020) downloaded from: http://cbi.hzau.edu.cn/rape/download_ext/zs11.genome.fa

4.2.6. Linkage map construction and QTL detection

Variants among the sequenced population were identified by comparing the aligned sequence with the high-quality *B. napus* reference genome ZS11 (Table S4.1) (Song et al. 2020) on the ArrayStar 16 (DNASTAR, Madison, WI, USA) platform. High-quality SNP sites in the population were identified by filtering SNP variants following the criteria of Depth coverage >5,

Quality score (Q) >10 and the SNP percentage >10% of the SNPs in the population. All of the monomorphic markers that did not differentiate between the parental genotypes, insertion-deletion markers, and any markers with error coding were removed from the data file. Moreover, any marker with > 20% missing bases was also removed from the marker genotype file. Linkage analysis among the markers and genetic map was constructed using JoinMap 4.1 (Ooijen and Voorrips 2001) with the filtered SNPs variants obtained from the population. The SNPs used in the linkage map were further analyzed and filtered on the QTL IciMapping (Meng et al. 2015) platform using 'binning' to remove redundant markers from the SNP file. The input file to run on QTL IciMapping was prepared by converting the SNP allele of the resistant parent genotype to '0', the susceptible parent genotype to '2', and the missing alleles to '-1' in the file. The QTL associated with resistance to pathotypes 3A, 3D, 3H, and 5X were identified using the input file. The segregation pattern of the markers and marker distortion was also calculated on the QTL IciMapping platform by performing the χ^2 test for goodness of fit. The QTL was declared based on the output of IciMapping run at 1,000-permutations with a type I error rate of 0.01. The suggestive QTL, QTL which exceeded the LOD threshold 3.0, was identified in association with tightly linked markers to the QTL and phenotypic variation caused by the QTL. The marker sequences in the QTL region were blasted with Blast2GO (Conesa et al. 2005) against the *Arabidopsi*s gene model, and candidate genes associated with clubroot resistance in the region were searched within the QTL.

4.2.7. Kompetitive Allele-Specific PCR (KASP) analysis

The previously identified SNP markers, linked to $Rcr9^{ECD02}$, on chromosome A08 of *B. rapa* and *B. napus* were used for KASP analysis. The analysis was performed following the manufacturer's instructions (http://www.lgcgroup.com/), with PCR carried out in a StepOne Plus

Real Time PCR System (Applied Biosystem, Mississauga, ON). Linkage analysis with the confirmed SNP markers and phenotypes based on the mean DSIs was performed using JoinMap 4.1 (Van Ooijen, 2001). The homologous position of the genes, mapped using ZS11, was identified on the A08 chromosome of the updated and widely used *B. rapa* genome v3.0 (chiifu). The markers developed from v3.0 chiifu were used to locate the homologous position on the A08 chromosome via KASP analysis.

4.3. Results

4.3.1. Population development and evaluation of resistance to pathotypes 3A, 3D, 3H and 5X

The parental lines DH16516 (Parent 1), 'AAFC695' (Parent 2), and their F_1 progeny were tested against pathotypes 3A, 3D, 3H and 5X of *P. brassicae.* Line DH16516 was highly susceptible (DSI = 100%) to all four pathotypes, whereas 'AAFC695' was highly resistant (DSI = 0 %). Lines Y12 and Y68 were also highly resistant (DSI = 0%) to all the pathotypes. In contrast, NRC11-24 and '45H29' were highly susceptible (DSI = 100%) to pathotypes 3A, 3D and 5X, but highly resistant to 3H (Table 4.1; Figure 4.1; Figures S4.1 & S4.2). Among the 102 lines of the DH population derived from the F_1 of the cross DH19516 × 'AAFC695', 52 were resistant and 50 were susceptible to pathotype 3A, while 51 were resistant and 51 were susceptible to pathotypes 3D, 3H and 5X. Segregation of the R and S reactions fit a 1:1 ratio for all the pathotypes (Table 4.1; Table S4.5; Figure 4.2). The DSI values of the lines in response to the pathotypes were also highly correlated in the population.

4.3.2. Statistical analysis of the phenotypic data

The sample means, variance, and standard error of the mean for the 102 DH lines were calculated on the QTL IciMapping (Table S4.2) platform to test the normality of the distribution

of the samples. The skewness of the sample mean and kurtosis were also calculated to check the quality of the distribution of the data. The skewness values ranged from 0.0 to 0.1, indicating that the data were symmetrical and normally distributed. The calculated skewness also showed that the data tended to lie in the middle, closer to the mean value without many outliers. The skewness values were -2 for all of the samples, indicating that the samples were platykurtic with a thinner tail, and that the data were normally distributed with very few outliers from the normally distributed data point (Table S4.2). The data sample ranged from 0 to 100.

4.3.3. DNA sequencing and genotyping

The two parents DH16516 and 'AAFC695' produced approximately 219.9 M (from WGS) and 7.4 M (from GBS) short reads, respectively. Approximately 79% of the short reads (depth coverage 18×) from DH16516 and 84% of the short reads (depth coverage 1.3×) from the parent 'AAFC695' were aligned to the reference genome ZS11. The parent DH16516 produced 9.02 M of SNPs (52.2 SNPs/1000 reads), and the reads covered 84.6% of the whole genome. In the same way, the parent 'AAFC695' genome showed 0.024 M SNPs, with 39.01 SNPs/1000 reads, and the aligned reads providing 4.21% coverage of the whole genome (Table S4.3). More than 520 M short reads were obtained from the population (102 lines), of which approximately 78% of the reads (~405 M) were aligned to the reference genome ZS11 on SeqMan NGen 16 (DNASTAR, Madison, WI, USA). The reads covered 3.72% of each templates, with 0.71-fold coverage of the whole genome. A total 2.12 M of SNPs were identified in the population, with approximately 52 SNPs per 1000 assembled short reads (Table S4.3).

4.3.4. Identification of polymorphic SNPs and construction of linkage maps

A total of 8,391,140 SNPs were detected in the whole population prior to filtering. Insertion-deletion, heterozygous and monomorphic markers from at least 50% of the population

(51 of 102 DH lines) were removed with the ArrayStar 16 (DNASTAR, Madison, WI) pipeline, leaving 58,859 SNP sites. After removing insertion-deletion, heterozygous and monomorphic markers, 5,652 high-quality SNPs were identified and run on the QTL IciMapping platform. The binning command of the QTL IciMapping removed an additional 3,170 redundant markers from the marker profile based on missing markers rate, anchor information, and missing values. This left 2,482 high-quality markers that were further filtered in Excel based on the interval between adjacent markers and missing SNPs rate, and the markers with > 25 cM distance were removed. Ultimately, 2,380 SNPs with intervals < 20 cM (Table S4.4) and a missing rate ≤ 3.77% were used for the QTL mapping. These 2,380 SNPs were distributed throughout 19 chromosomes with an average of 125 SNPs on each chromosome (Table S4.4; Figure 4.3). The SNPs covered 2138.77cM of the whole genome with an average length of 112.57 cM per chromosome (Table S4.4) and an average distance of 1.47 cM between adjacent markers.

4.3.5. QTL Mapping

A single QTL, *Rcr9*^{AAFC695}, associated with SNP markers ZS_A08_18117664 and ZS_A08_20072807 on linkage group A08, was identified for resistance to the four tested pathotypes 3A, 3D, 3H, and 5X (Figure S4.3). This QTL explained 92.6% of the phenotypic variation in resistance to pathotype 3A, with an additive effect (independent effect of each allele at a locus) of 47.2% and a LOD value of 60.3 (Table 4.3; Figure 4.4). *Rcr9*^{AAFC695} also explained 95.0%, 94.9%, and 93.4% of the resistance to pathotypes 3D, 3H, and 5X, respectively, with additive effects of 48.7%, 48.7%, and 47.7% corresponding to LOD values of 70.8, 70.4, and 63.3 (Table 4.3; Figure 4.4). The identified QTL spanned between 76.74 cM and 83.44 cM on the linkage group A08. The peak position was found at 82.0 cM of the linkage group, where the

nearest flanking marker ZS-A08_18117664 was located at 82.45 cM of the linkage group A08 (Table 4.3; Figure 4.4).

4.3.6. QTL analysis and candidate gene annotation

The coding sequences (cds) from the 1.96 Mb region between the two flanked markers ZS_A08_18117664 and ZS_A08_20072807 were pulled from the reference genome, without going beyond the flanking markers, and run on the Blast2GO and NCBI Blast platforms to identify candidate genes. There were 283 *B. napus* genes identified in the 1.96 Mb QTL region of the reference genome, of which two, *BnaA08T0141000ZS* (ZS_A08_19001038 ─ ZS_A08_19002195) homologous to *BraA08g017130.3C* (*B. rapa* v3.0 Chiifu) and *BnaA08T0142200ZS* (ZS_A08_19068453 ─ ZS_A08_19071577) homologous to *BraA08g016670.3C* (*B. rapa* v3.0 Chiifu), were NBS_LRR (Nuclear Binding Site-Leucine Rich Repeat) genes (Table 4.4) encoding disease resistance proteins. None of the RLP and RLK genes were identified in the region.

4.3.7. KASP analysis

Kompetitive Allele-Specific PCR was performed to genotype the 102 DH lines with the 33 markers associated with resistance to the different pathotypes, which had previously been identified on *B. napus* and chromosome A08 of *B. rapa* (Figure 4.5 & 4.6). Among these markers, nine (CF_A08_ 11385371, CF_A08- 11627096, CF_A08_10850975, CF_A08_10855744, CF_A08_11038256, CF_A08_11059924, CF_A08_11219809, CF_A08_11672817, and CF_A08_11855997) were found to co-segregate with the QTL $Rcr9^{AAFC695}$ flanked by the left and right markers ZS_A08_18117664 and ZS_A08_20072807 of the ZS11 reference genome.

4.3.8. Comparing the identified QTL region with *B. rapa* V3.0 'Chiifu' reference genome

As the *B. napus* reference genome ZS11 has not been widely used to map *CR* genes in the Brassicas, the identified QTL $Rcr9^{AAFC695}$ position was compared with the A08 chromosome of *B. rapa* genome v3.0 (Chiifu). Chromosome A08 of *B. rapa* is a hot spot of identified *CR* genes (Hasan & Rahman 2016; Hirani et al. 2018; Yu et al. 2021). The identified 1.96 Mb QTL region flanked by the left and right markers ZS_A08_18117664 and ZS_A08_20072807 in ZS11 was homologous to a 2.0 Mb region on the A08 chromosome of *B. rapa* genome v3.0 (Chiifu) (Figure 4.6)

4.4. Discussion

The deployment of genetically resistant canola is one of the most effective, environmentally friendly and convenient methods to manage clubrooot. This study was carried out to identify and map the clubroot resistance in the *B. napus* cultivar 'AAFC695', which is strongly resistant to some of the new resistance-breaking pathotypes of *P. brassicae* (Chapter 2; Dakouri et al. 2021). A DH population developed from the F_1 progeny of a cross between 'AAFC695' and the accession DH16516 showed a typical bimodal distribution [1:1 (R:S)] for segregation of resistance to pathotypes 3A, 3D, 3H and 5X, while the F_1 plants were highly resistant (DSI ~0%). These findings suggest the involvement of a major dominant gene. In addition, the resistance reactions to each of the pathotypes were highly correlated (r = 0.97-0.99), indicating the control of resistance by a single gene or cluster of tightly linked genes. In a previous study, a similar distribution was observed in a rutabaga-BF (*B. napus*) population and a major gene was identified on chromosome A08, which conferred resistance to pathotypes 2, 3, 5, 6, and 8 (Hasan & Rahman 2016) as defined on the differentials of Williams (1966).

Genotype-by-sequencing produced 520.7 M reads from the population, with 58,859 SNPs that were used to identify and map the *CR* genes by the construction of a robust linkage map. A single QTL (*Rcr9*^{AAFC695}) was mapped, which showed more than 90% PV with > 47% additive effects for the tested pathotypes 3A, 3D, 3H, and 5X. The positive additive values confirmed the presence of the resistance gene in the resistant parent (Yu et al. 2017), 'AAFC695', used in the study. The location of the identified QTL was homologous to the 11.9 to 13.9 Mb region of chromosome A08 in *B. rapa* 'Chiifu' v3.0 (Figure 4.7). This QTL was named *Rcr9*^{AAFC695} because it was identified in the same genetic region where *Rcr9* was mapped in a *B. rapa* breeding line T19 (Yu et al. 2017). *Rcr9* was shown to confer resistance to pathotype 5X (Yu et al. 2017), but not pathotypes 3A, 2B, 3D (Chapter 2) or 3H (Yu et al. 2017). In contrast, *Rcr9*^{AAFC695} bestowed resistance to all of the pathotypes in this study, including 5X. This suggests that *Rcr9*^{AAFC695} is not the same as *Rcr9*. Moreover, the *Rcr9* genomic region spanned a relatively large interval (6.48 Mb) on chromosome A08, where some other *CR* QTL/genes, including *Rcr3,* *Rcr9*^{DBR}, *Rcr9*^{wa,} and *Rcr9*^{ECD02}, have previously been identified and mapped (Yu et al. 2017; Hasan & Rahman 2016; Karim et al. 2020; Yu et al. 2021; Rahaman et al. 2022)

The QTL *Rcr3* mapped to the 11,385,371-11,627,069 Mb region of chromosome A08 of *B. rapa* v3.0, which is beyond the region where *Rcr9*^{AAFC695} was identified in this study. Moreover, *Rcr3* conferred resistance to pathotype 3H but not 5X (Karim et al. 2020), indicating that it is different from *Rcr9*^{AAFC695}. The *Rcr9*^{wa} was identified in the *B. rapa* European Clubroot Differential (ECD) 04, in a region spanning 12.3-12.6 Mb of chromosome A08 and conferring resistance to pathotype 5X (Karim et al. 2020); the resistance of this gene to pathotypes 3A and 3D was not tested. Nonetheless, the genetic position of *Rcr9*^{wa} was slightly smaller than for *Rcr9*^{AAFC695}, suggesting that *Rcr9*^{wa} and *Rcr9*^{AAFC695} are not the same gene. This should be

confirmed by testing *Rcr9*^{wa} against pathotypes 3A and 3D, and fine mapping and cloning the resistance gene(s) in the QTL. Another QTL, *Rcr9*^{ECD01}, which is distinct from *Rcr9* (Yu et al. 2021), was identified on the A08 chromosome of *B. rapa* ECD 01, in a similar region to *Rcr9*^{AAFC695}. However, while *Rcr9*^{ECD01} conferred resistance to pathotypes 3D, 3H and 5X, which were also used to map *Rcr9*^{AAFC695} in this study, resistance to pathotype 3A was controlled by the genes in two QTL *Rcr9*^{ECD01} and *Rcr10* ^{ECD01} (Yu et al. 2021). In contrast, *Rcr9*^{AAFC695} alone conferred resistance to 3A in the current analysis. Therefore, *Rcr9*^{AAFC695} appears to be different from *Rcr9*^{ECD01} (Figure 4.7).

In *B. rapa* ECD 01, *Rcr9*^{ECD01} was identified using the reference genome 'Darmor-*bzh*', while *Rcr9*^{AAFC695} was identified in *B. napus* using the reference genome ZS11. Both of the genetic regions were compared to the location in the *B. rapa* genome v3.0 and corresponded to the region where *Rcr9* was mapped. While both of these QTL are in the same *Rcr9* genomic region, the location on v3.0 differs slightly (*Rcr9*^{DBR}: 12.0 to 14.5 Mb of chromosome A08; *Rcr9*^{AAFC695}: 11.9 to 13.9 Mb of A08), and the QTL were identified in two different species (*B. rapa,* and *B. napus*). The QTL/gene *Rcr9*^{ECD02} was also found in the *B. rapa* genotype ECD 02 and conferred resistance to pathotypes 3A, 3D, 3H, and 5X of *P. brassicae*; however, the *NBS-LRR* genes identified in the QTL were different. The two *NBS-LRR* genes (*BnaA08T0141000ZS* and *BnaA08T0142200ZS*) identified in the *Rcr9*^{AAFC695} region, which were homologous to *BraA08g017130.3C* and *BraA08g016670.3C* in *B. rapa* chiifu v3.0, were different from the four *NBS-LRR* genes in *Rcr9*^{ECD02}. Another major gene, *CRs* (11.3 – 12.2 Mb), was identified in the *Rcr9* genomic region and conferred resistance to pathotype 4 (Laila et al. 2019) as classified on the differentials of Williams (1966). There is no information, however, on the reaction of *CRs* to the new pathotypes 3A, 3D, 3H, or 5X. In the same way, a major CR locus was identified in the

12.1-13.0 Mb region of the rutabaga A08 chromosome (Hasan & Rahman 2016) that conferred resistance to Williams' pathotypes 2, 3, 5, 6, and 8, but which has not been tested against pathotypes 3A, 3D, 3H, or 5X. A major QTL was also identified in the 9.96-11.09 Mb region of A08 (Wang et al. 2022), which was effective against pathotypes 3H, 3A, and 3D. This QTL, however, only showed a non-allelic resistance reaction with another QTL on chromosome A03. Therefore, since only a non-allelic interaction of genes from A03 and A08 resulted in resistance to the pathotypes, further analysis is needed to make any conclusions regarding the similarities between these QTL and the identified genes.

The gene *BraA.CR.b* was also identified on chromosome A08 of the *B. rapa* hosts ECD 01, ECD 02, ECD 03 and ECD 04, and conferred resistance to pathotype 3H (Hirani et al. 2018). However, there is no information regarding the corresponding location of this gene in the *B. rapa* v3.0 genome. Therefore, comparisons between *BraA.CR.b* and $Rcr9^{AAFC695}$ are not possible at present, but could be the focus of future studies. Another QTL termed *qBrCR38-2*, which conferred resistance to race 7 of *P. brassicae*, was identified in the 20.3-21.8 Mb region of chromosome A08 (Zhu et al. 2019), far from the currently identified $Rcr9^{AAFC695}$. The cloned CR gene *Crr1* is also located in the *Rcr9* region, but a breeding line carrying only *Crr1* was susceptible to pathotypes 3A, 3D, 3H, and 5X (Yu et al. 2021). Thus, based on the phenotypic reaction of the gene to the pathotypes, it could be concluded that *Crr1* is not the same as $Rcr9^{AAFC695}$ in 'AAFC695'. This must be confirmed, however, by sequencing of the gene.

Plant *NBS-LRR* genes represent the largest class of disease resistance genes and are involved in the detection of many plant pathogens, including *P. brassicae* (Akira and Hemmi 2003). Within this class, TIR-domain containing NBS-LRR proteins (TNL) trigger host resistance against biotrophic pathogens via induction of the hypersensitive response,

programmed cell death (apoptosis), and signal transduction (Nandety et al. 2013) (Ueno et al. 2012; Hatakeyama et al. 2013). The two *NBS-LRR* genes identified in the *Rcr9*^{AAFC695} region were of the TNL subclass, and would be expected to provide strong resistance to *P. brassicae*, as observed in this study, unless targeted by effectors produced by specific pathotypes. They could be fine-mapped and cloned for further characterization and use in clubroot resistance-breeding activities.

4.5. Conclusion

The current study identified and mapped a major clubroot resistance locus effective against four resistance-breaking pathotypes of *P. brassicae*, 3A, 3D, 3H, and 5X, which have recently emerged in western Canada. The segregation ratio in the 102 DH lines was 1:1 resistant:susceptible, with the reactions to the pathotypes found to be highly correlated. Collectively, these results indicate that the clubroot resistance in the *B. napus* genotype 'AAFC695' is controlled by a major gene or tightly linked genes. A high throughput GBS approach was used to construct a high-resolution genetic map and to identify and map the resistance genes. A single QTL, *Rcr9*^{AAFC695}, was detected and mapped to the 1.96 Mb region of chromosome A08 of 'AAFC695'. KASP analysis identified nine markers that cosegregated with *Rcr9*^{AAFC695} . The QTL region identified was homologous to the 11.9-13.9 Mb region on the A08 chromosome of *B. rapa* genome V3.0 (Chiifu). Among the 283 genes identified in the QTL region, two *NBS-LRR* class genes encoded disease resistance proteins. These genes could be cloned and transformed into canola to confirm their role. Ultimately, *Rcr9*^{AAFC695} may be an important resource in the development of canola cultivars with improved resistance to clubroot in western Canada and other regions. Its identification in a *B. napus* background would facilitate introgression into commercial *B. napus* canola cultivars.

4.6. Tables

Table 4. 1**.** Segregation patterns in *Brassica napus* 'AAFC695' doubled-haploid (DH) lines, derived from the F_1 of DH16516 × AAFC695, their parents and the F_1 generation in comparison with the resistant control lines Y12 and Y68, and the susceptible controls NRC11-24, '45H29', and DH16516.

Lines/ crosses	Pathotypes/ number of plants							
	3A		3D		3H		5X	
	R	S	R	S	R	S	R	S
'AAFC695' (P_2)	12	0	29	0	12	0	12	0
DH16516 (P_1)	0	18	0	84	0	13	0	13
F_1 (DHT × 'AAFC695')	13	0	12	0	12	0	12	0
Y12	25	0	29	0	30	0	24	0
Y68	6	0	-	-	-	-	-	-
NRC11-24	0	12	0	16	-	-	0	12
45H29	-	-	0	13	-	-	-	-
'AAFC695' DH	51	51	51	51	51	51	50	52
$\chi 2$	0.00		0.00		0.00		0.00	
Calculated p-value	0.370		1.00		1.00		0.804	
P-value (at 0.05, 1df)	3.84		3.84		3.84		3.84	

Table 4. 2**.** Correlations in reactions of a *Brassica napus* 'AAFC695' doubled-haploid population, derived from the F_1 of DH16516 × AAFC695, to pathotypes 3A, 3D, 3H, and 5X of *Plasmodiophora brassicae*.

Pathotypes	3A	3D	3H	5X
3A	1			
3D	0.986**	1		
3H	0.984**	0.997**	1	
5X	0.978**	0.990**	0.990**	1

** Significance level at P≤0.01.

Table 4. 3**.** Identified QTL and the phenotypic effect explained by the QTL in a *Brassica napus* 'AAFC695' doubled-haploid population, derived from the F_1 of DH16516 × AAFC695, in response to pathotypes 3A, 3D, 3H, and 5X of *Plasmodiophora brassicae*.

Pathotypes	QTL/gene	Chrom.	Position	Left Marker	Right Marker	LOD	PVE (%)	Add.(%)
3A	*Rcr9*^{AAFC695}	8	82	ZS_A08_18117664	ZS_A08_20072807	60.3	92.6	47.2
3D	*Rcr9*^{AAFC695}	8	82	ZS_A08_18117664	ZS_A08_20072807	70.8	95.0	48.7
3H	*Rcr9*^{AAFC695}	8	82	ZS_A08_18117664	ZS_A08_20072807	70.4	94.9	48.7
5X	*Rcr9*^{AAFC695}	8	82	ZS_A08_18117664	ZS_A08_20072807	63.3	93.4	47.7

Table 4. 4**.** Identified TIR-NB-LRR (TNL) genes in *Rcr9*^{AAFC695} on chromosome A08 of a *Brassica napus* 'AAFC695' doubled-haploid population, derived from the F_1 of DH16516 × AAFC695. The genes were identified based on Blast2Go and NCBI output against the Arabidopsis gene model.

Gene name	*B. napus* (ZS11) gene location (base)	Length (base)	Gene description from Blast2Go and NCBI	Homolog in *B. rapa* (v3.0 Chiifu)	*R* gene class
BnaA08T0141000ZS	19001038 ─ 19002195	1157	Disease resistance protein (TIR-NBS-LRR class) family	*BraA08g017130.3C*	NBS-LRR class
BnaA08T0142200ZS	19068453 ─ 19071577	3124	Disease resistance protein (TIR-NBS-LRR class) family	*BraA08g016670.3C*	NBS-LRR class

4.7. Figures

DHT (P1) **'AAFC695' (P2)** **DHT × 'AAFC695' (F1)**

Figure 4. 1**.** Clubroot symptom development on resistant and susceptible parents and their F_1 progeny; DHT = DH16516 (susceptible parent), 'AAFC695' = winter oilseed rape 'AAFC695' (resistant parent), and DHT × 'AAFC695' = F_1 progeny of DH16516 × AAFC695.

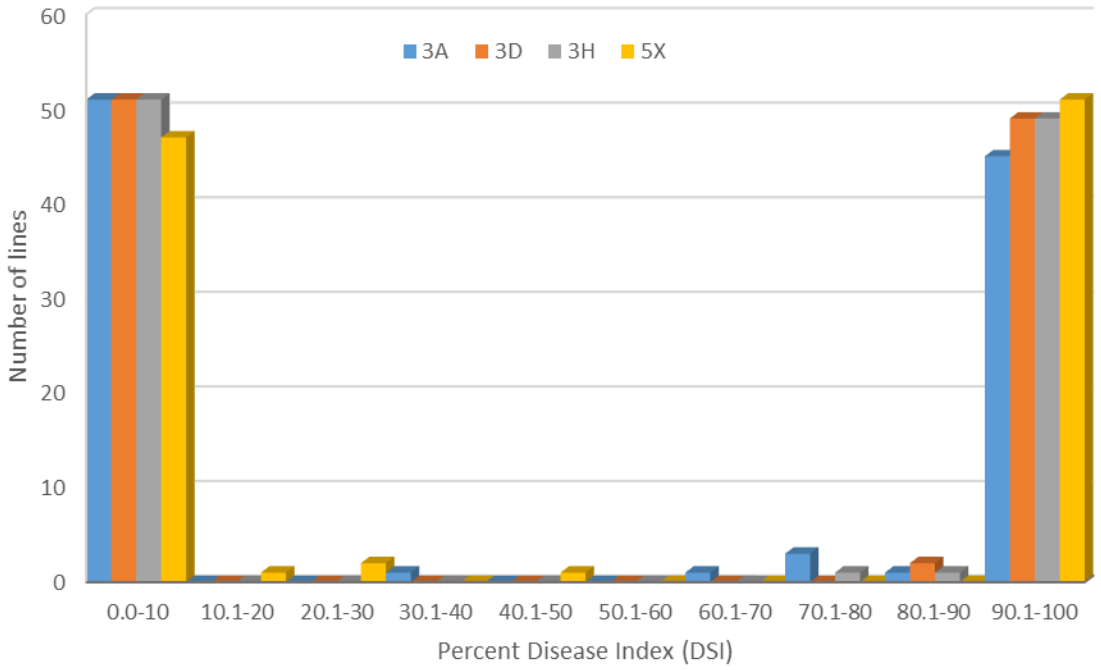


Figure 4. 2**.** Clubroot disease severity index (DSI, %) on 102 *Brassica napus* 'AAFC695' doubled haploid lines, derived from the F_1 of DH16516 × AAFC695, in response to inoculation with pathotypes 3A, 3D, 3H, and 5X of *Plasmodiophora brassicae*.

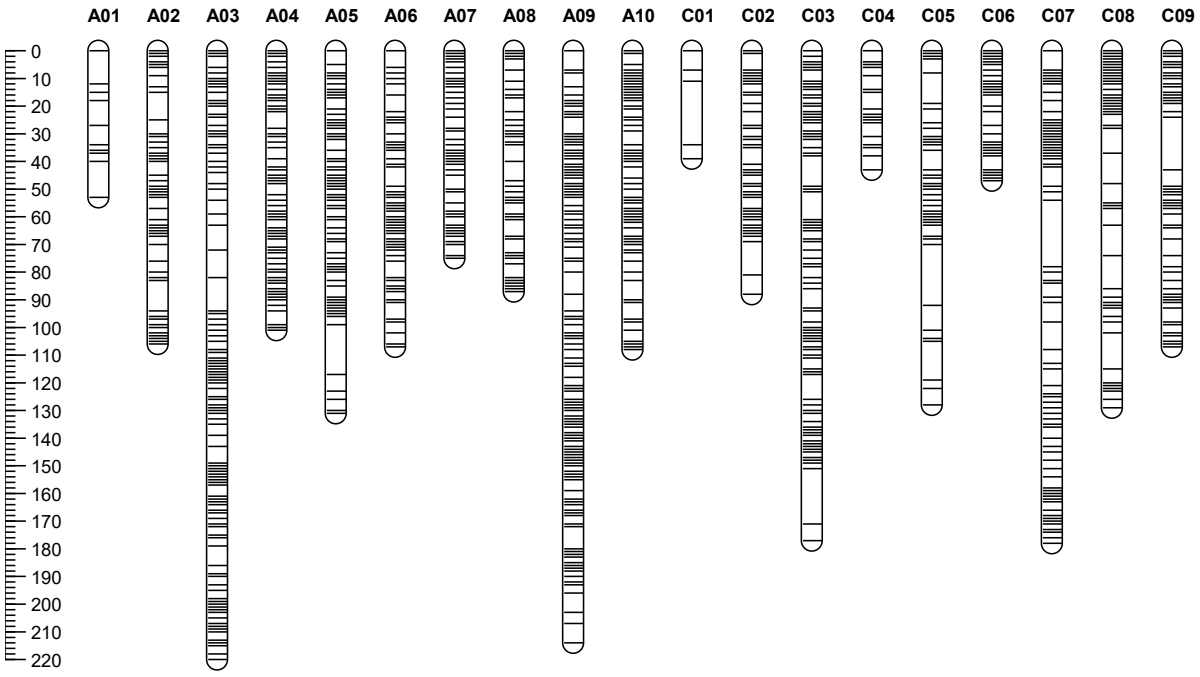


Figure 4. 3**.** Genetic linkage map of a *Brassica napus* doubled haploid population, derived from the F_1 of DH16516 × AAFC695, consisting of 2,380 markers distributed on 19 chromosomes with an average distance of 1.47 cM between the adjacent markers. The vertical scale on the left indicates the genetic distance in centiMorgan (cM).

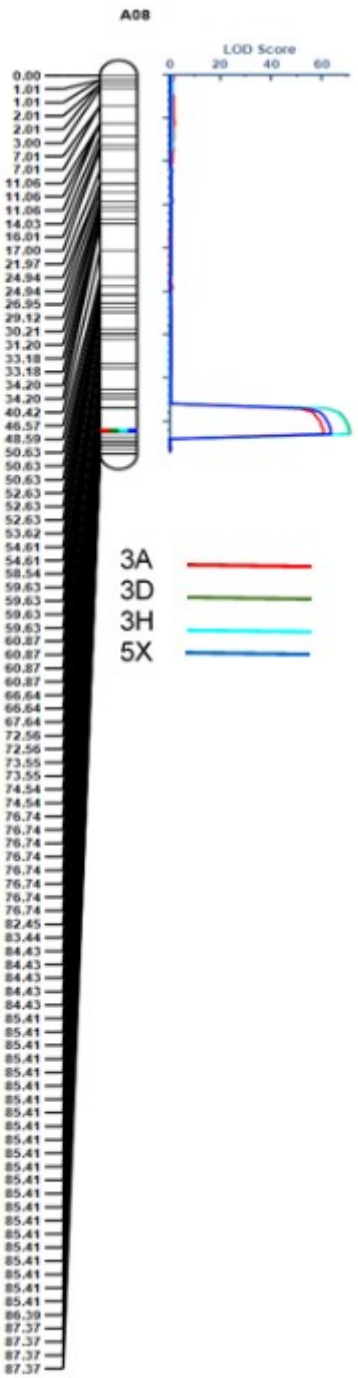


Figure 4. 4**.** The complete linkage map of chromosome A08 and the significant QTL position identified in the linkage group in a *Brassica napus* 'AAFC695' doubled-haploid population derived from the F_1 of DH16516 × AAFC695.

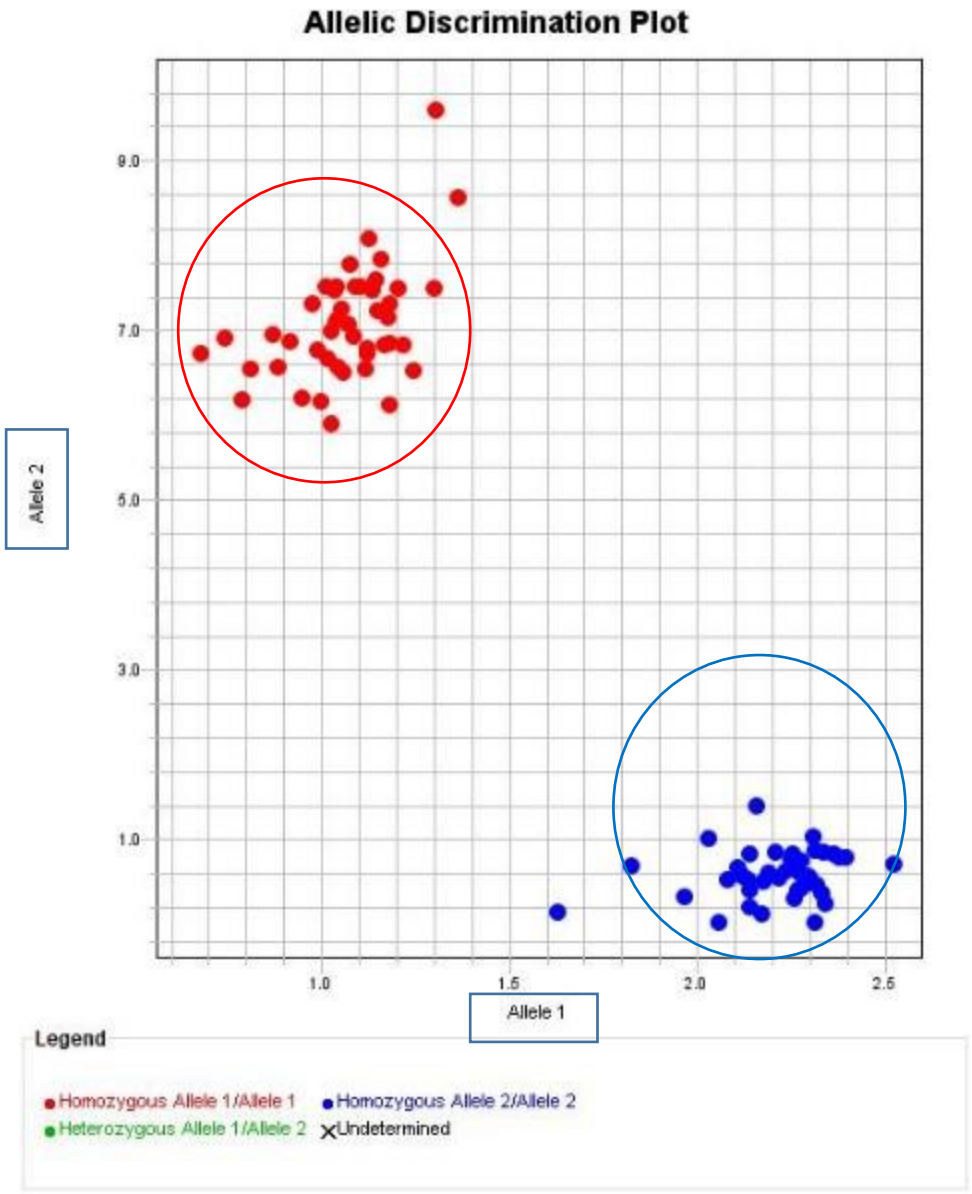


Figure 4. 5. Allelic discrimination plot from KASP SNP markers analysis of 102 doubled haploid lines developed from the F_1 progeny of DH16516 × 'AAFC695' of *Brassica napus*. The red cluster indicates the homozygous alleles from susceptible lines and the blue cluster indicates the homozygous alleles from resistant lines.

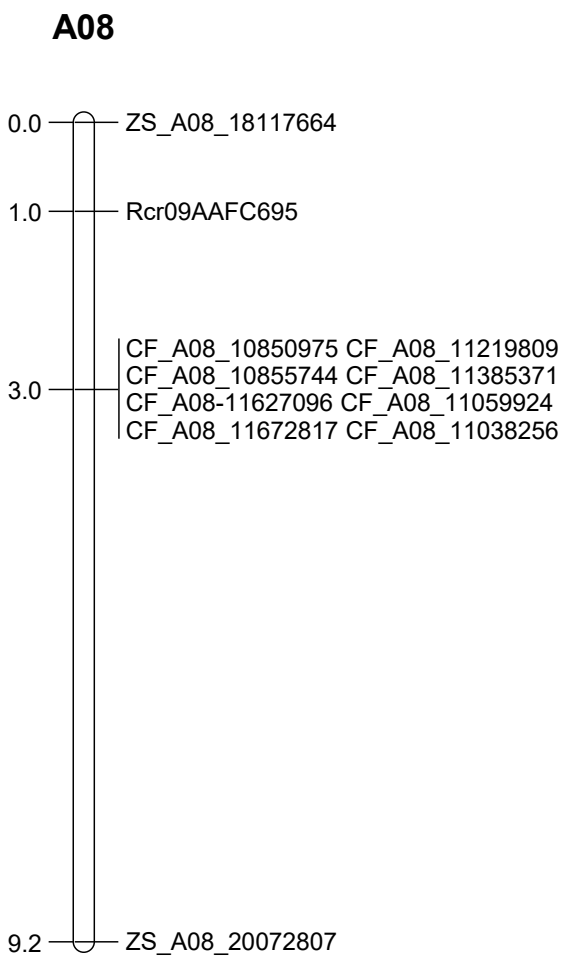


Figure 4. 6. A linkage map drawn from the KASP marker analysis to link SNPs identified from *Brassica rapa* in the *Brassica napus* genotype 'AAFC695'. CF indicates the SNP markers developed using *B. rapa* genome v3.0 (Chiifu); ZS-indicates the SNP markers identified using *B. napus* reference genome ZS11.

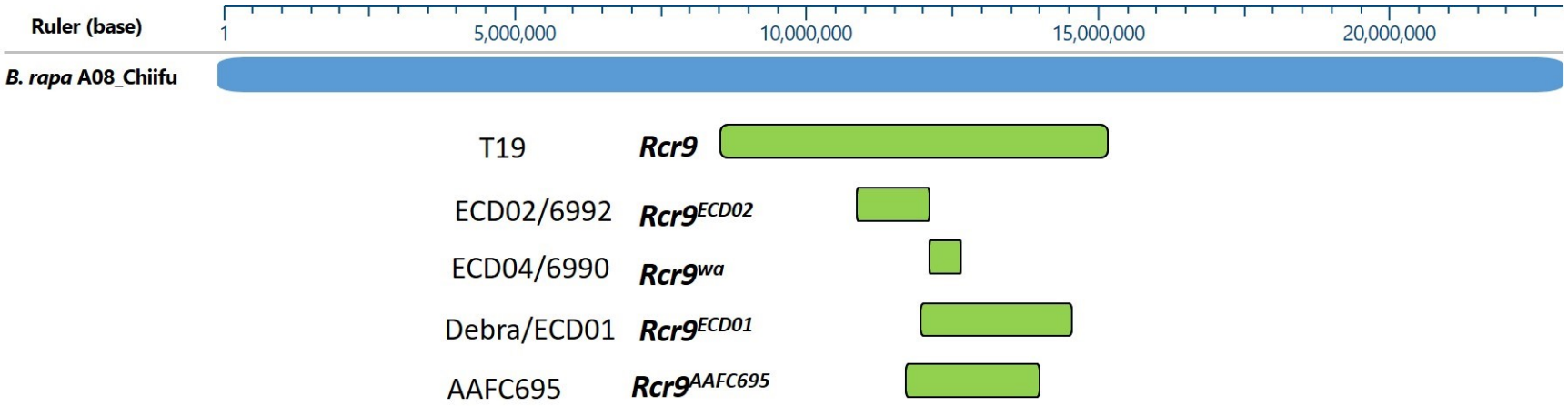


Figure 4. 7. Corresponding location of the identified QTL *Rcr9*^{AAFC695} on A08 chromosome of *B. rapa* genome v3.0 (Chiifu) in relation to the previously identified QTL *Rcr9* (Yu et al. 2017), *Rcr9*^{ECD02} (Rahaman et al. 2022), *Rcr9*^{wa} (Karim et al. 2020), and *Rcr9*^{ECD01} (Yu et al. 2021) from different populations.

Chapter 5. Identification of a second gene for resistance to four pathotypes of *Plasmodiophora brassicae* in the *Brassica napus* cultivar 'Mendel'

5.1. Introduction

Canola or rapeseed (primarily *Brassica napus* L.) is an oilseed crop grown in temperate regions worldwide to produce heart-healthy edible oil. The global production of edible oil from canola is second only to soybean (Kupiec et. al. 2020; USDA 2020). Canada is one of the major canola-producing countries, and this crop contributes an estimated $29.9 billion CAD annually to the national economy (Canola Council of Canada, 2020). Clubroot, caused by the obligate parasite *Plasmodiophora brassicae*, is an important soilborne disease of canola and other cruciferous crops. The management of clubroot is challenging, as the pathogen produces long-lived resting spores that can persist in the soil for many years, and the disease can cause yield losses as high as 30–100% under conducive conditions (Tewari et al. 2005; Strelkov et al. 2007). Since the first identification of clubroot on the Canadian Prairies in 2003, the disease has continued to spread (Strelkov & Hwang 2014; Gossen et al. 2015), with more than 3300 field infestations confirmed by 2020 (Strelkov et al. 2020; Hollman et al. 2021). Fortunately, genetic resistance to clubroot, used in combination with crop rotation, can reduce clubroot severity (Rahman et al. 2014; Peng et al. 2014; Strelkov et al. 2016) for successful production of canola.

Clubroot-resistant (CR) canola cultivars were first released in Canada in 2009–2010 and were highly effective against all of the major pathotypes of *P. brassicae* that had been identified up to that point (Strelkov et al. 2016). However, severe symptoms of clubroot were observed on CR canola in two fields in Alberta in 2013 (Strelkov et al. 2016). The isolates recovered from these fields were classified as pathotype 5 based on the differential system of Williams (1966),

but differed from other pathotype 5 isolates in their ability to overcome the CR trait; hence, isolates with this new virulence profile were designated as pathotype 5X (Strelkov et al. 2018). Many more fields with resistance breakdown have been identified in Alberta since 2013, and the Canadian Clubroot Differential (CCD) set was developed to characterize the pathotypes responsible (Strelkov et al. 2018). In the CCD system, isolates are assigned a number based on their Williams' classification, followed by a letter to indicate their CCD designation (Askarian et al. 2021; Strelkov et al. 2018). This enables the identification of multiple variants of the same Williams' pathotype.

The identification of novel *P. brassicae* pathotypes able to overcome the resistance in CR canola cultivars indicated the need for novel sources of resistance and/or pyramiding of resistance genes (Rahman et al. 2014; Yu et al. 2016). *Brassica rapa* is an important source of clubroot resistance (Buczacki et al. 1975; Hatakeyama et al. 2017). More than 20 CR loci/genes have been mapped to specific chromosomes in *B. rapa*, including chromosome A03: *CRa, CRb, CRd, CRk, Crr3, PbBa3.1, PbBa3, Rcr1, Rcr2, Rcr4,* and *Rcr5* (Matsumoto et al. 1998; Piao et al. 2004; Pang et al. 2018; Sakamoto et al. 2008; Hirai et al. 2004; Saito et al. 2006; Chen et al. 2013; Chu et al. 2014; Huang et al. 2017; Yu et al. 2017; Huang et al. 2019); chromosome A08: *Crr1, CRs, Rcr9, Rcr3, Rcr9wa,* $Rcr9^{DBR}$, $Rcr9^{ECD02}$ (Suwabe et al. 2003; Laila et al. 2019; Yu et al. 2017; Karim et al. 2020; Yu et al. 2021; Rahaman et al. 2022); chromosome A02: *CRc, Rcr8* (Sakamoto et al. 2008; Yu et al. 2017); chromosome A06: *Crr4* (Suwabe et al. 2006); and chromosome A01: *Crr2* (Suwabe et al. 2006). In addition, numerous QTL involved in clubroot resistance, many of which correspond to genes identified in *B. rapa,* have been reported in *B. napus* (Piao et al. 2009; Manzanares-Dauleux et al. 2000; Werner et al. 2008; Piao et al. 2004; reviewed in Piao et al. 2009).

Single nucleotide polymorphisms (SNPs) have been widely used as molecular markers to identify QTL and resistance genes, as well in studies of genetic variation, genetic map construction, population structure analysis, association studies and map-based gene cloning (Kumar et al. 2012). Genotyping-by-sequencing (GBS) is a simple and promising tool that enables the inexpensive and rapid discovery of SNP variants in *Brassica* species (Yu et al. 2017) and many other crops. Genetic markers from GBS provide a high-throughput but precise technology to map clubroot resistance genes in *Brassica* genotypes, including the winter *B. napus* cultivar 'Mendel'.

'Mendel' was developed for resistance to clubroot from a re-synthesized *B. napus* line by crossing *B. oleracea* line ECD15 (CC, 2*n* = 18) with *B. rapa* line ECD04 (AA, 2*n* = 20) (Diederichsen and Sacristan, 1996). The A genome in 'Mendel' (from ECD04) carries the clubroot resistance (Diederichsen et al. 2006; Fredua-Agyeman & Rahman 2016; Hirani et al. 2018). The gene in 'Mendel' for resistance to the initial group of pathotypes known in Canada was linked to the clubroot resistance loci/genes *CRk*, *Crr3*, and *CRb* (Fredua-Agyeman & Rahman 2016) on chromosome A03. Genetic mapping of CR loci/ genes from the ECD04-derived line 96-6990 led to the identification of two tightly linked CR loci on chromosome A08, including *Rcr3* for resistance to pathotype 3H and $Rcr9^{wa}$ for resistance to pathotype 5X (Karim et al. 2020). 'Mendel' was resistant to 13 of 17 pathotypes of *P. brassicae* identified in Alberta and so was included as a differential in the CCD set (Strelkov et al. 2018).

This study was undertaken to identify and map any gene(s) present in 'Mendel' for resistance to selected new and clubroot resistance-breaking pathotypes of *P. brassicae*, and to develop SNP markers tightly linked to the *CR* gene(s).Tightly-linked SNP markers could be used

to improve selection for clubroot resistance that is effective against newly identified pathotypes of *P. brassicae* in Canada.

5.2. Methods

5.2.1. Plant materials and DH development

A study was carried out to identify the genes responsible for the clubroot resistance of *B. napus* cv. 'Mendel'. The study was initiated by crossing 'Mendel' with DH16516, a doubled haploid (DH) line of *B. napus* provided by Dr. G. Séguin-Swartz (Agriculture and Agri-Food Canada (AAFC), Saskatoon Research and Development Centre, Saskatoon, SK); DH16516 was derived from the clubroot-susceptible *B. napus* cv. 'Topas'. 'Mendel' is a winter type, so it was vernalized in a growth chamber under controlled conditions (4°C;16 hr light/ 8 hr dark) for 8 weeks to induce flowering. Following vernalization, the plants were returned to normal growth conditions (21°C/18°C) in the greenhouse for flowering. The susceptible parent, DH16516, was spring type in nature, and hence seeding was timed so that greenhouse-grown plants flowered at the same time as 'Mendel'. To prevent unintended pollination, plants were covered with a transparent plastic pollination bag at the time of bud initiation. The F_1 progenies were produced from the cross between the two parental lines, and the F_1 plants were assessed for clubroot reaction.

5.2.2. DH population development

A DH population from three clubroot-resistant F_1 plants from the DH16516 × 'Mendel' cross was developed via microspore culture (Coventry et al. 1988, cited by Hasan & Rahman 2016). As described in Chapter 4, three F_1 plants were grown in a growth chamber set at 10°C with a 14 h light /10 h dark cycle. Buds of 3.5-4.5 mm in length were collected in a 50 mL Falcon tube (Glendale, AZ) partially filled with ice to protect the microspores from degeneration

and to inhibit meiosis. The isolated buds were surface-sterilized in a 3% calcium hypochlorite ($Ca(OCl)_2$) solution for 10 min, then rinsed three times with cold sterile Milli-Q Millipore water. The sterilized flower buds were then homogenized in 25 mL liquid Gamborg B5 medium (Gamborg et al. 1968) with a mortar and pestle. The homogenate was filtered through two nested layers of sterile filters [64 µm top (NTX64) and 41 µm bottom (NTX41)] in a sterile 15 mL Falcon tube (Corning). The tube containing the pellet was centrifuged at 1000 rpm for 3 min and the supernatant was discarded. The pellet was washed three times with B5 washing medium. The cleaned microspore pellet was re-suspended in 25 mL NLN 13 medium (Lichter 1982 cited by Hasan & Rahman 2016) with 50 mg/L colchicine and the suspension was poured into sterile Petri dishes (8.9-cm diam.) and then sealed with parafilm.

The Petri dishes containing the microspores were incubated in darkness at 32°C for 72 h, then transferred to an incubator set at 30°C for 14 days. Afterward, the dishes were placed in a paper box that was sealed to exclude light and placed on a shaker rotating at 60 rpm for 3 days. The Petri dishes were then removed from the sealed box and exposed to a 16/8 h day/night regime to allow the embryos to grow.

The embryos were grown under light until they developed a torpedo shape at about 8 days, and were then transferred to a refrigerator at 4°C for 2–7 days. The embryos were then transferred again, to Petri dishes containing solid B5 medium and incubated at room temperature (21°C ± 2°C) with 16/8 h day/night until plantlets began to develop. When the seedlings produced well-developed roots and true leaves, they were transplanted into pots filled with a soil-free growth medium (Sunshine Mix 3, TerraLink Horticulture Inc., Abbotsford, BC) and covered with transparent cups for 1 week to maintain the high humidity needed to encourage strong growth. The cups were then removed and the plantlets were exposed to ambient

conditions and allowed to grow until flowering. Plants that produced visible pollen were considered doubled haploids, while plants with smaller flowers/petals and lacking pollen grains in the flower were considered to be haploid and were discarded. Seeds from each of the remaining 137 DH lines were harvested and stored for phenotyping.

5.2.3. Phenotyping

Evaluation of plants for resistance to clubroot was conducted in the growth chambers and greenhouse of the AAFC Saskatoon Research and Development Center from 2018 to 2020. The assessment method was similar to that of Yu et al. (2021) with some modifications. In all, the reactions of 137 DH lines developed from microspore culture were assessed. In addition, two clubroot-resistant DH *B. napus* lines carrying the CR loci $Rcr9^{ECD01}$ and $Rcr10^{ECD01}$ (AAFC-Y12 and AAFC-Y68) (Yu et al. 2021), and the clubroot-susceptible DH line DH16516, were included as controls.

'Mendel' has been reported to be resistant to 13 pathotypes of *P. brassicae*, including 5C, 3D, 8E, 2F, 5G, 3H, 5I, 8J, 5K, 5L, 6M, 8N, and 3O (Strelkov et al. 2018). As the identification of *CR* genes has been conducted previously in the ECD04-derived line 96-6990 with pathotypes 3H and 5X (Karim et al 2020), and in 'Mendel' with pathotypes 2F, 3H, 5I, 6M and 8N (Hasan & Rahman 2016; Fredua-Agyeman & Rahman 2016), the clubroot reaction to the three other pathotypes, 5C, 3D, and 8J, was initially evaluated in this study. After identifying QTL for these three pathotypes in all 137 DH lines, the clubroot reaction of a subset of DH lines to pathotype 3H also was assessed.

Inoculum of pathotype 3H was produced in the *B. napus* line DH16516. Inoculum of pathotypes 5C, 3D, and 8J was produced in the *B. napus* line NRC11-24 (Nutrien Ag. Solutions, Saskatoon, Saskatchewan), which is resistant to pathotype 3H but susceptible to pathotypes 3A,

3D and 5X (C. Franke, personal communication; Yu et al. 2021). Six weeks after inoculation of the seedlings, the infected roots were harvested and stored at -20°C for preparing the inoculum for the phenotyping study.

Inoculum was prepared following Karim et al. (2020). For each pathotype, about 100 g of frozen galled roots was soaked into 200 mL of water for 1 h and then homogenized in a blender for 2–3 min. The homogenized suspension was strained through two layers of nylon cloth, and the spore concentration in the filtrate was estimated with a hemocytometer and then diluted to 1 $\times 10^7$ resting spores mL^{-1} with water. The resting spore suspension was stored at -20°C until needed for inoculation. Seedlings were grown in 10 cm × 10 cm plastic plant pots in trays containing 32 pots. The pots were filled with saturated soilless mix (Sunshine Mix 3, TerraLink Horticulture Inc.). Any extra water was drained off the tray. Each pot was seeded with 6–9 seeds of a single line, and each tray included one resistant and one susceptible line as a control. Immediately after seeding, the seeds were covered with the potting mix, compacted to ensure good seed-to-growth mix contact, and the tray was covered with a transparent lid to minimize drying. The trays were placed in a growth chamber at 21°C ± 2°C with a 16-h photoperiod for 7 days to allow the seeds to germinate and the seedlings to produce well-developed root hairs.

Each pot was then inoculated with 15 mL of thawed inoculum suspension and the trays were covered with transparent lids for another 2 weeks. After the lids were removed, the seedlings were watered from the bottom of the tray. Seedlings were maintained in a greenhouse until five weeks after inoculation, then uprooted, washed, and scored for clubroot symptoms on a 0–3 scale (Kuginuki et al. 1999), where: 0 = no galls, 1 = a few small galls, 2 = moderate galls, and 3 = severe galling on the roots (Figure S5.1). The individual ratings were used to calculate a disease severity index (DSI) according to the formula of Horiuchi and Hori (1980) as modified

by Strelkov et al. (2006), and as described in Chapter 2 of the thesis. The evaluated seedlings were classified as resistant or susceptible based on the DSI (%), where a DSI ≤ 30% was regarded as resistant (R) and a DSI >30% as susceptible (S). The inheritance pattern of resistance to the tested pathotypes was calculated by the Chi-Square ($\chi 2$) test for goodness of fit.

Each line with a resistant (DSI ≤ 30%) reaction in the initial study was re-assessed twice more. 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.

5.2.4. DNA sequencing and short reads assembly

DNA samples from the 137 DH lines and the two parents, 'Mendel' and DH16516, were extracted from young seedlings with a DNeasy Plant Mini Kit (Qiagen, Toronto, ON) following the manufacturer's instructions. The quality and concentration of the extracted DNA were determined with a Qubit Fluorometer, NanoDrop (Thermo ScientificTM) and Microplate Reader. The sample integrity was evaluated by gel electrophoresis. The extracted DNA was disgested with a methylation-sensitive restriction enzyme ApeKI to reduce the complexity of the DNA and to prepare the DNA library for sequencing. The fragmented DNA, produced from the digestion, was ligated to enzyme-specific adapters and amplified through PCR (Dakouri et al. 2018). GBS was performed on the 137 DH lines and parent 'Mendel' on an Illumina platform with pair-end sequencing at BGI Americas Corp (Cambridge, MA, USA). The susceptible parental line DH16516 was sequenced at the Plant Biotechnology Centre (Saskatoon, SK, Canada). The sequenced short reads were aligned with SeqMan NGen 16 (DNASTAR, Madison, WI) following standard filtering parameters (DNASTAR, Madison, WI, USA; Yu et al. 2017) using

the *B. napus* reference genome ZS11, which was downloaded from: http://cbi.hzau.edu.cn/rape/download_ext/zs11.genome.fa.

5.2.5. Variant identification, linkage map construction, and QTL detection

GBS-SNP variants of the sequenced population were identified in comparison with the *B. napus* reference genome ZS11 (Song et al. 2020) using SeqMan NGen 16, and the GBS-SNP sites were compared using ArrayStar 16 (DNASTAR). The identified SNP sites in the population were filtered by setting the criteria of Depth coverage > 5, Quality score (Q) > 10, and the SNP percentage > 10%.

The SNP alleles identified from each resistant DH line were scored with '0', the susceptible DH lines were scored with '2', and missing values were scored with '-1' for use of the SNPs file in QTL IciMapping (Meng et.al. 2015) for the detection of QTL associated with the trait. QTL IciMapping was also used to construct the linkage map and to examine the Mendelian segregation distortion of each marker through a χ^2 test. The maximum likelihood in Kosambi's model with a minimum LOD value of 3.0 was used to order the marker positions on the map, and the map was drawn with Mapchart 2.1 (Voorrips, 2002) based on the marker position on the genetic map determined by QTL IciMapping. QTL IciMapping was also used to identify and declare the QTL with a minimum LOD threshold of 3.0 with 1,000-permutations. The IciMapping program was run at 1,000-permutations, called bootstrapping, with a type I error rate of 0.01 to declare the QTL. The QTL for a particular trait and the effect of the QTL on the particular trait were estimated based on the phenotypic variation explained (PVE) and additive (Add) effect caused by the QTL.

5.2.6. Gene tagging in the targeted QTL region

The coding sequences (CDS) associated with the QTL mapped by IciMapping were annotated against the Arabidopsis gene model to identify candidate genes using Blast2GO (Conesa et al. 2005). Genes in the region associated with disease resistance, signal transduction associated with disease resistance, and plant defense response were identified from the Blast2Go output. The RLP, RLKs, and TIR-NBS-LRR genes in the region were searched as candidate genes, given their widely reported roles in plant disease resistance.

5.2.7. Kompetitive Allele-Specific PCR (KASP)

KASP analysis was performed to validate the loci/genes identified through GBS, as well as to identify more SNPs in the QTL region. Markers identified from different populations of *B. rapa* and *B. napus* on chromosome A08 in previous studies were used to run the KASP and genotype the entire population. Tightly linked marker(s) were identified and a genetic linkage map was constructed using joinMap 4.1. Since most of the genes or QTL for clubroot resistance have been identified from *B. rapa*, the corresponding position of the identified QTL was mapped on the current and updated version of the *B. rapa* reference genome 'Chiifu' v3.0 using the previously developed markers from *B. rapa* chromosome A08 (Yu et al. 2017; Karim et al. 2020; Yu et al. 2021).

5.3. Results

5.3.1. Clubroot resistance of the parental lines and the F_1

In total, 137 DH lines were developed from the F_1 progeny of a cross between DH16516 × 'Mendel'. The reaction of the DH lines, the parents 'Mendel' and DH16516, the F_1 progeny, and the three lines used as controls (AAFC-Y12, AAFC-Y68 and NRC11-24) to pathotypes 5C, 3D, and 8J was evaluated. The susceptible parent DH16516 and the susceptible control line

NRC11-24 were highly susceptible to all three pathotypes (100% DSI), whereas 'Mendel', the resistant control lines AAFC-Y12 and AAFC-Y68 and the F_1 plants were highly resistant to all three pathotypes (0% DSI) (Table 5.1). The DSI values for all three pathotypes were highly correlated. However, the segregation pattern for resistance among the DH lines differed for the three pathotypes (Table S5.3). The Chi-square (χ^2) test indicated a 3:1 segregation ratio of resistant (R) to susceptible (S) for all of the three pathotypes (Table 5.1; Figure S5.2).

5.3.2. GBS analysis and SNP identification

GBS short-read sequence analysis of the resistant parental cultivar 'Mendel' generated 0.99 M (million) short-read sequences, of which 0.88 M reads were aligned (depth coverage 0.2×) with the reference genome ZS11 of *B. napus*. The aligned reads were approximately 89% of the total read sequences. There were 40,000 SNPs identified in the aligned sequences with ~45 SNPs/ 1000 assembled reads. There were 219.9 million (M) short reads generated from the susceptible parental line DH16516, of which approximately 92.1% (202.5 Million) of the total number of short reads were aligned with the reference genome (depth coverage 21×). The aligned read sequences provided ~91.1% of coverage of the whole genome. A total of 11.9 M SNPs were identified within the assembled sequences with ~63.3 SNPs/ 1000 read sequences. The 137 DH lines generated ~704.5 M short reads, of which ~635.6 M (~90.2%) reads were aligned with the reference genome (Table S5.1). A total of ~37.9 M SNPs were identified from the assemblies with ~60.0 SNPs/1000 assembled short reads.

5.3.3. Variant analysis and construction of a linkage map

After filtering on Array Star, 81,453 SNPs remained, which were further reduced by removing insertion-deletion, heterozygous, monomorphic markers, and those with missing SNPs >20% using Microsoft Excel. The remaining 2,642 tightly linked markers were distributed

among the 19 chromosomes, with an average of 139 SNPs per chromosome. The whole genome was 5834.7 cM with an average of 307.1 cM per chromosome. The average missing rate of the remaining SNPs was 4.5%, and the average distorted markers were ~111 markers per chromosome (Figure 5.2; Table S5.2).

5.3.4. QTL detection and mapping

QTL mapping was performed on QTL IciMapping (Meng et al. 2015) in association with the filtered and tightly-linked markers and phenotype (DSI) values of the population for pathotypes 5C, 3D and 8J. A single QTL for resistance to pathotypes 3D, 5C, and 8J on chromosome A08 (located close to gene *Rcr3*; Karim et al. 2020) was identified and named *Rcr3*^{Mendel} (Table 5.3; Figure S5.3). The QTL position was tagged with flanking markers ZS_A08_15999175 (left marker) and ZS_A08_16316110 (right marker). The LOD, PVE and Add values for *Rcr3*^{Mendel} varied among the pathotypes, ranging from 63 to 68 for LOD, 88 to 90% for PVE, 47.2 to 47.3% for Add, and 86.4 to 90.5 cM for CI (Table 5.3)

The identified QTL was positioned on linkage group A08, with a confidence interval (CI) of 116.7 to 117.6 (Table 5.3; Figure S5.3). One of the flanking markers, ZS_A08_15999175, was located at the position of the QTL. The values of the Additive effect for the QTL were positive, indicating that the resistance loci were derived from the resistant parent 'Mendel'.

5.3.5. Candidate gene identification

Blast2Go and NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to search for candidate genes in the *Rcr3*^{Mendel} QTL region. The coding sequences (CDS) from the 0.3 Mb region between the flanking markers ZS_A08_15999175 (left marker) and ZS_A08_16316110 (right marker) were used as the query sequences. Eighty-two candidate genes were identified in

the region, including a single TIR_NBS_LRR (TNL) gene *BnaA08T0102200ZS* (ZS_A08_16222504 – ZS_A08_16225680) homologous to *BraA08g016670.3C* in *B. rapa* v3.0 (Chiifu) (Table 5.4). None of the RLP or RLK genes were found in the region.

5.3.6. KASP analysis and validation of identified QTL

The position of the identified QTL was validated using 40 KASP primers developed from *B. rapa* and *B. napus* that had been linked to *CR* genes identified on the A08 chromosome. In this KASP, 16 markers co-segregated with *Rcr3*^{Mendel}. A linkage map was constructed with the co-segregated markers and six markers were tightly linked to the *Rcr3*^{Mendel} QTL: A08_ZS_16705472, A08_CF_12275702, A08_CF_11021839, A08_CF_11466518, A08_CF_12063499, and A08_CF_12230973 (Table 5.4; Figure S5.4). The two tightly linked markers (A08_CF_11021839, A08_CF_11466518) that co-segregated with *Rcr3*^{Mendel} had previously been shown to co-segregate with *Rcr3* in *B. rapa* (Karim et al. 2020).

5.3.7. Identified QTL position corresponding to *B. rapa* reference genome v3.0 (Chiifu)

The region associated with the identified QTL, *Rcr3*^{Mendel}, was compared and aligned against the most recent version of the *B. rapa* reference genome v3.0 (Chiifu) to identify its corresponding position on the reference genome. A 0.3 Mb region of the *Rcr3*^{Mendel} QTL on chromosome A08 of *B. napus* was homologous to the region of 11.0-12.3 Mb in *B. rapa* (Figures 5.4 and 5.5).

5.3.8. *Rcr3*^{Mendel} for resistance to pathotype 3H

Rcr3 was identified for resistance to pathotype 3H, while *Rcr3*^{Mendel} was identified by testing for resistance to pathotypes 5C, 3D and 8J. A subset of the population consisting of 60 of the 137 DH lines in the DH population was tested for resistance to 3H, and it was observed that

resistance to 3H (Table S5.4; Figure S5.5) co-segregated with the resistance phenotypes against pathotypes 5C, 3D and 8J. The result indicates that *Rcr3*^{Mendel} is likely the same as *Rcr3*.

5.4. Discussion

In the current study, a gene responsible for the resistance of the *B. napus* cv. 'Mendel' to pathotypes 3D, 5C, and 8J of *P. brassicae* was identified and mapped through GBS and KASP analysis from a segregating DH population consisting of 137 lines from F_1 plants. These pathotypes were selected for study because the resistance in 'Mendel' had not been genetically mapped with them previously. 'Mendel' was previously reported as resistant to the initial cohort of clubroot pathotypes identified on the Canadian Prairies (Fredua-Agyeman & Rahman 2016; Hasan & Rahman 2016) and was used to develop the first generation of CR canola cultivars (Rahman et al. 2011; Hasan & Rahman 2016; Fredua-Agyeman et al. 2018). It was also resistant to many of the new resistance-breaking pathotypes, including 3D, 5C, and 8J (Strelkov et al. 2018).

The DSI values of the DH lines to pathotypes 3D, 5C, and 8J were highly correlated suggesting that the traits were likely controlled by a major gene or a cluster of tightly linked genes (Yu et al. 2017; Karim et al. 2020). The resistant lines showed strong, consistent resistance to all three pathotypes (DSI = 0%), which supported the hypothesis that resistance was controlled by a major gene (Karim et al. 2020; Yu et al. 2021; Rahaman et al. 2022). The segregation ratio of the DH lines was used to analyze the segregation pattern of the gene and to estimate the number of gene(s) associated with the trait. Based on a threshold DSI of 30% (Yu et al. 2021), a 3:1 ratio (R:S) was observed in the DH population in response to inoculation with pathotypes 3D, 5C and 8J. This threshold to distinguish resistant from susceptible reactions is more stringent than for out-crossed phenotypes, which are more variable than DH lines. The 3:1 ratio also

supports the hypothesis that the resistance trait is likely controlled by more than one, independently segregating gene (Yu et al. 2017; Yu et al. 2021). However, only one resistance QTL/gene was identified for all of the tested pathotypes. This could reflect segregation distortion of the traits in the population through microspore culture, potentially due to embryogenesis in favor of the resistance allele.

A single QTL was identified as the major QTL responsible for resistance to the three pathotypes. This QTL was named *Rcr3*^{Mendel} because it mapped in 'Mendel' to a similar region of chromosome A08 where the *Rcr3* had been identified in the *B. rapa* 'Wasalander' (ECD04) (Karim et al. 2020). *Rcr3*^{Mendel} was tagged in the 316.9 kb region of the A08 chromosome, flanked by the left marker ZS_A08_15999175 and right marker ZS_A08_16316110; the nearest marker was ZS_A08_15999175.

The reaction to pathotype 3H was subsequently assessed in 60 of the DH lines, since this had been the pathotype used originally to identify *Rcr3*. Resistance to 3H co-segregated with resistance to pathotypes 5C, 3D and 8J, indicating that *Rcr3*^{Mendel} was likely the same as *Rcr3*. In total, 42 genes were identified in the *Rcr3*^{Mendel} region, but only one gene, *BnaA08T0102200ZS* (ZS_A08_16222504 – ZS_A08_16225680), was identified as a *TIR_NBS_LRR* (TNL) gene; TNL genes play a significant role in resistance to clubroot (Akira and Hemmi 2003; Wang et al. 2022; Kopec et al. 2021), by inducing disease resistance proteins (Nandety et al. 2013; Kopec et al. 2021) and signal transduction (Ueno et al. 2012; Hatakeyama et al. 2013; Wang et al. 2022). The TNL gene identified in this study is homologous to *AT5G51630* in Arabidopsis and *BraA08g016670.3C* in *B. rapa* (Chiifu v3.0).

Segregation distortion (deviation of observed genotypic frequencies from the expected frequencies) has been observed in many crop species, including Brassica crops (Kianian and

Quiros, 1992; Cloutier et al. 1995; Zhang et al. 2003). The level of distortion depends on the plant species, population, and the parental lines used in the crosses (Prince et al. 1993; Lu et al. 2002). DH populations, especially those generated by androgenesis from microspore culture, often show more extreme segregation distortion than F_2 or backcross populations (Lashermes et al. 2001). The 'Mendel' population was developed by androgenesis (microspore culture) and, therefore, the extreme segregation could be caused by environmental influences or chromosome instability of the microspores. Several researchers have found that environmental influences, recombination, transgenic silencing (Pawlowski et al. 1998), chromosome instability (Tonguc et al. 2003), pollen tube competition, and competitive fertilization with gametic or zygotic selection pressure (Kreike and Stiekema, 1997; Xian-Liang et al. 2006) would be driving forces for segregation distortion by altering the gamete ratio by the segregation distortion locus (SDL) of the population.

Segregation distortion of the SNP loci on chromosome A08 was observed in the DH population (Table S5.2). Since segregation distortion is a common phenomenon in the evolution of many crop species, removal of the segregation distortion markers could reduce genome coverage by the markers, and QTL might be missed in linkage mapping (Xu et al.1997; Taylor and Ingvarsson 2003; Zhang et al. 2003). Nonetheless, while segregation distortion was observed in the population, plants were found that showed strong resistance (DSI~0) to the tested pathotypes. This indicates that the trait was controlled by a single dominant gene in the host. Similar strong resistance reaction results were obtained previously for the clubroot resistance QTL/genes *Rcr1–Rcr7* (Yu et al. 2017; Chu et al. 2014; Huang et al. 2017; Huang et al. 2019; Yu et al. 2016; Karim et al. 2020; Dakouri et al. 2018; Chang et al. 2019).

GBS can be used to find large numbers of SNPs throughout the genome (Lee et al. 2016; Mundada et al. 2022; Rajendran et al. 2022), which are in turn used to identify and map QTL/genes for a specific trait. While GBS can produce erroneous SNP calling and missing data points (Spindel et al. 2013), stringent filtering of the data is applied to minimize the error and identify high-quality SNPs (Spindel et al. 2013; Yu et al. 2017). The technology has been widely used to map genes and QTL in various crop species including maize, wheat, rice, barley, soybean, cotton, broccoli, cabbage, and canola (Elshire et al. 2011; Crossa et al. 2013; Romay et al. 2013; Poland et al. 2012; Spindel et al. 2013; Sonah et al. 2013; Qi et al. 2017; Branham et al. 2017; Lee et al. 2016; Yu et al. 2017; Karim et al. 2020; Mundada et al. 2022; Wang et al. 2020; Rajendran et al. 2022). A high-quality reference genome, denoted as ZS11 (Song et al. 2020), was used in this study to assemble and map the QTL/gene. GBS in combination with KASP analysis is a highly effective approach to mapping QTL (Chapter 3; Karim et al. 2020).

The identified QTL *Rcr3*^{Mendel} region was homologous to the 11.0–12.3 Mb of chromosome A08 in the *B. rapa* reference genome v3.0 (Chiifu). Many clubroot resistance genes have been identified and mapped in the A genome of *B. rapa*, so an updated version (v3.0) of the *B. rapa* genome (Zhang et al. 2018) was used to identify the corresponding location of *Rcr3*^{Mendel}. In that assessment, *Rcr3*^{Mendel} was homologous to the region of *B. rapa* v3.0 where other *CR* genes and QTL have been identified, including *Rcr9* (Yu et al. 2017), *Rcr9*^{ECD01} (Yu et al. 2021), *Rcr9*^{wa} (Karim et al. 2020), *Rcr9*^{ECD02} (Chapter 3), *Rcr9*^{AAFC695} (Chapter 4), and *Rcr3* (Karim et al. 2020) and a major QTL in Rutabaga (Hasan & Rahman 2016, 2021; Wang et al. 2022). The differential reaction to several pathotypes in the current study suggests that *Rcr3*^{Mendel} is different from *Rcr9*; the latter provided resistance to pathotype 5X but not to 3H (Yu et al. 2017), while *Rcr3* provided resistance to 3H. In addition, the QTL region associated with *Rcr9*

spanned a larger interval (6.48 Mb) than the QTL for *Rcr3*. Similarly, $Rcr3^{Mendel}$ is not the same as $Rcr9^{ECD01}$ because $Rcr9^{ECD01}$ was identified as conferring resistance to pathotypes 3A, 3D, 3H, and 5X (Yu et al. 2021; Chapter 4), while 'Mendel', the resistant genotype used to map $Rcr3^{Mendel}$, is susceptible to pathotypes 3A and 5X (Strelkov et al. 2018). Furthermore, $Rcr9^{AAFC695}$ (Chapter 4) is not the same QTL/gene as $Rcr3^{Mendel}$ (Table 5.5 and Table 5.6). There is another QTL $Rcr9^{ECD02}$ that was also identified in the *Rcr9* QTL region and found to confer resistance to pathotypes 3A, 3D, 3H, and 5X, but again, 'Mendel' is susceptible to pathotypes 3A and 5X. Hence, $Rcr3^{Mendel}$ cannot be the same QTL as $Rcr9^{ECD02}$ (Table 5.5 and Table 5.6; Figure 5.5).

Finally, the *B. rapa* genotype ECD04, which was one of the diploid progenitors of the *B. napus* cv. 'Mendel', is resistant to pathotype 5X (Karim et al. 2020) while 'Mendel' is susceptible (Strelkov et. al. 2018). The QTL $Rcr9^{wa}$, which confers resistance to pathotype 5X, was identified and mapped in the *Rcr9* region of ECD04 (Karim et al. 2020). Therefore, the resistance gene identified in 'Mendel' in this study is likely different from $Rcr9^{wa}$. Possible reasons why the $Rcr9^{wa}$ does not occur in 'Mendel' is that the gene was not inherited during hybridization between ECD04 × ECD15. A previous study demonstrated that the *CR* gene *Crr1* was also lost or not inherited from ECD04 at the time of hybridization to develop 'Mendel' (Agyeman et al. 2018).

Still other CR loci/genes have been identified in the A08 chromosome of *B. rapa* and *B. napus,* and their relationship to $Rcr3^{Mendel}$ is not known. For example, *CRs* lies in a similar region to both *Rcr9* and *Rcr3* and conferred resistance to pathotype 4 (Laila et al. 2019). However, no data are available regarding its influence on the reaction to pathotypes 3D, 5C, and 8J. Similarly, a major QTL resistance to pathotypes 2, 3, 5, 6, and 8 were identified in the *Rcr9*

region in Rutabaga (Hasan & Rahman 2016), but no information regarding the resistance reaction to the new pathotypes 3D, 5C, and 8J. Further studies are required to be able to make inferences regarding the genetic position of *CRs* and the QTL identified in Rutabaga relative to the currently identified *Rcr3*^{Mendel}. The gene *BraA.CR.b* was mapped in ECD01, ECD02, ECD03 and ECD04 and conferred resistance to pathotype 3H (Hirani et al. 2018). While the gene was identified on the A08 chromosome, its position on *B. rapa* genome v3.0 is not known, making it difficult to compare its position with that of *Rcr3*^{Mendel}. In contrast, the clubroot resistance QTL *qBrCR38-2*, conferring resistance to pathotype 7, was identified at 20.3– 21.8 Mb (Zhu et al. 2019) of chromosome A08, far from the position of *Rcr3*^{Mendel}.

Finally, the cloned gene *Crr1* has also been mapped to the *Rcr9* QTL region of chromosome A08. *Crr1* gene did not confer resistance to four major pathotypes of *P. brassicae* from Canada, including 3H (Yu et al. 2021); in contrast, *Rcr3*^{Mendel} provided resistance to 3H, and hence (based on this resistance reaction), *Rcr3*^{Mendel} must be different from *Crr1*.

Rcr3^{Mendel} was identified as a major gene for resistance to pathotypes 3D, 5C, and 8J with 88-90% phenotypic variation against the pathotypes. Any QTL that shows a consistent PVE of >10% is termed a major QTL (Wang et al. 2019; Yu et al. 2021) for that trait. Moreover, the DSIs in response to the three pathotypes among the DH lines were highly correlated, further indicating that the trait is controlled by a major gene or a cluster of tightly linked genes (Yu et al. 2017; Yu et al. 2021). This study was designed and conducted to map *CR* genes through rough mapping. Fine mapping with a larger population size is needed to determine whether a single *CR* gene or cluster of tightly linked genes controls the resistance reaction in this system.

5.5. Conclusion

A population consisting of 137 DH lines was developed from the winter *B. napus* cv. 'Mendel' and used to identify and map *CR* genes effective against pathotypes 3D, 5C, and 8J of *P. brassicae*. A single, resistance QTL, *Rcr3*^{Mendel}, was identified and mapped by genotyping-by-sequencing and KASP analysis, showing 88–90% resistance to pathotypes 3D, 5C, and 8J. A subset of the population consisting of 60 lines were used to determine if *Rcr3*^{Mendel} was the same as *Rcr3*, which had been identified previously. *Rcr3*^{Mendel} was positioned in the 316.9 kb region of the A08 chromosome flanked by the left marker ZS_A08_15999175 and right marker ZS_A08_16316110. KASP analysis identified eight more tightly linked markers that co-segregated with *Rcr3*^{Mendel}. *Rcr3*^{Mendel} also co-segregated with *Rcr3*, which conferred resistance to pathotype 3H. *Rcr3*^{Mendel} was homologous to the 11.0 to 12.3 Mb region in the *B. rapa* genome Chiifu. Among the 82 genes identified in the *Rcr3*^{Mendel} region, only one, *BnaA08T0102200ZS* (ZS_A08_16222504 – ZS_A08_16225680), was identified as a TNL gene. The identified region of *Rcr3*^{Mendel} overlapped with the QTL region of *Rcr9*^{ECD01}, *Rcr9*^{wa}, *Rcr9*^{ECD02,} and *Rcr9*^{AAFC695}, but there are several lines of evidence that indicate that it is different from those QTL. Further investigation, including fine mapping and cloning of the gene, is needed to characterize *Rcr3*^{Mendel} fully.

5.6. Tables

Table 5.1**.** Segregation patterns of a *B. napus* doubled haploid (DH) population derived from the parental genotypes 'Mendel' and DH16516, two resistant checks (AAFC-Y12 and AAFC-Y68), and a DH line NRC11-24 resistant to *Plasmodiophora brassicae* pathotype 3H and susceptible to pathotypes 3D, 5C, and 8J.

Pathotypes	DSIs					No. of DH lines		$\chi 2$ (Calculated)	*P*-value))
	Mendel	DHT	NRC 11-24	Y12	Y68	R	S	3:1	
3D	0	100	100	0	0	107	30	0.63	0.43
5C	0	100	100	0	0	108	29	0.98	0.32
8J	0	100	100	0	0	110	27	1.92	0.17

Table 5.2**.** Correlation coefficient for clubroot severity (disease severity index) in doubled haploid (DH) lines, derived from F_1 of *Brassica napus* DH16516 × 'Mendel', and inoculated with pathotypes 3D, 5C and 8J of *Plasmodiophora brassicae*.

Pathotypes	3D	5C	8J
3D	1.00		
5C	0.97**	1.00	
8J	0.97**	0.98**	1.00

**Significance at $P \leq 0.01$

Table 5.3**.** Estimated position of *Rcr3*^{Mendel} on chromosome A08 of the *Brassica napus* cv. 'Mendel', with the corresponding LOD value, phenotypic variation explained (PVE), additive effect, and flanking markers associated with the gene in doubled haploid (DH) lines inoculated with *Plasmodiophora brassicae* pathotypes 3D, 5C and 8J.

Pathotype	QTL	LOD	PVE (%)	Add. (%)	Left markers	Right markers	Left CI	Right CI
3D	*Rcr3*^{Memdel}	63	88.4	47.2	ZS_A08_15999175	ZS_A08_16316110	86.4	90.5
5C	*Rcr3*^{Memdel}	66	89.2	47.3	ZS_A08_15999175	ZS_A08_16316110	86.4	90.5
8J	*Rcr3*^{Memdel}	68	90.1	47.2	ZS_A08_15999175	ZS_A08_16316110	86.4	90.5

Table 5. 4. TIR-NBS-LRR class resistance gene identified in the *Rcr3*^{Mendel} region of *Brassica napus* cv. Mendel associated with resistance to *Plasmodiophora brassicae* pathotypes 3D, 3H, 5C and 8J.

Gene name	*B. napus* (ZS11) gene location (base)	Length (base)	Gene description from Blas2Go and NCBI	Homolog in *B. rapa* (v3.0-Chiifu)	R gene class
BnaA08T0102200ZS	16222504---16225680	3176	Disease resistance protein RPP4-like	*BraA08g016670.3C*	*TIR-NBS-LRR* class

Table 5. 5. Identified QTL/genes in the *Brassica napus* genotypes 'AAFC695' and 'Mendel' and the *Brassica rapa* genotype ECD02, with corresponding genes and their positions in the A08 linkage group of *B. rapa* reference genome v3.0 (Chiifu). The LOD value, PVE, additive effect, and flanking markers associated with the QTL are also listed in the corresponding pathotypes.

Pathotypes	QTL	Chrom	Position	LOD	PVE (%)	Add. (%)	Left Marker	Right Marker
3D, 5C, 8J	*Rcr3*^{Memdel}	A08	87	63-68	88-90	40-47	ZS_A08_15999175	ZS_A08_16316110
3A, 3D, 5X, 3H	*Rcr9*$^{'AAFC695'}$	A08	82	60-70	92-95	47-48	ZS_A08_18117664	ZS_A08_20072807
3A, 3D, 3H, 5X	*Rcr9*^{ECD02}	A08	401	24-30	68-77	64-74	CF_A08_11903476	CF_A08_10575267

Table 5. 6. Disease resistance genes identified in the QTL *Rcr3*^{Memdel} and *Rcr9*$^{'AAFC695'}$ and their homologous genes in the *Brassica rapa* genome (v3.0. Chiifu).

QTL	Identified genes in *B. napus* (ZS11)	Gene location (ZS11/ v3.0 Chiifu)	Length (base)	Gene description (Blas2Go/ NCBI)	Homolog in *B. rapa* (v3.0 Chiifu)	Gene class
Rcr3^{Memdel}	*BnaA08T0102200ZS*	16222504─16225680	3176	Disease resistance protein RPP4-like	*BraA08g016670.3C*	TIR-NBS-LRR class
Rcr9^{AAFC695}	*BnaA08T0141000ZS*	19001038─19002195	1157	TIR-NBS-LRR class	*BraA08g017130.3C*	NBS-LRR class
	BnaA08T0142200ZS	19068453─19071577	3124	TIR-NBS-LRR class	*BraA08g016670.3C*	NBS-LRR class
Rcr9^{ECD02}	-	11232393─11235117	2724	receptor-like protein 30	*BraA08g012910.3C*	disease resistance
	-	11248087─11250727	2640	receptor-like protein 30	*BraA08g012920.3C*	disease resistance
	-	11388577─11391460	2883	receptor-like protein 30	*BraA08g013130.3C*	disease resistance
	-	11696216─11696785	569	a typical TIR-NBS-LRR	*BraA08g013630.3C*	disease resistance

Table 5. 7**.** List of identified clubroot resistance genes/QTL on the A08 chromosome of *B. rapa* and *B. napus*.

Cultivar/species	Identified genes/QTL	Type	Physical position	Pathotypes	References
B. rapa ssp. *chinensis* cv. Akimeki	*CRs*	Major gene	11.3 – 12.2 Mb	4	Laila et al. 2019
B. rapa ssp. *rapifera* cv. Waaslander	*Rcr3*	Gene	11.4 – 11.6 Mb	3	Karim et al. 2020
B. rapa ssp. *rapifera* cv. Siloga	*Crr1a*	Major gene	12.3 – 12.3 Mb	2	Swabe et al. (2003; Hatakeyama et al. 2013
B. rapa ssp. *rapifera* cvs. Pluto & Waaslander	*Rcr9/Rcr9^{wa}*	QTL	12.3 – 12.6 Mb	5X	Yu et al. 2017; Karim et al. 2020
B. rapa ssp. *rapifera* cv. Siloga	*Crr1b*	Gene	….	2	Hatakeyama et al. 2013
B. rapa ssp. *chinensis* cv. (Pakchoi)	*qBrCR38-2*	QTL	20.3 – 21.7 Mb	Race 7	Zhu et al. 2019
B. napus var. *napobrassica* cv. Brookfield	Single gene	Major gene	12.1 – 13.0 Mb	2, 3, 5, 6 & 8	Hasan & Rahman, 2016
B. rapa turnip cv. ECD01	*Rcr9^{ECD01}*	QTL	12.0 – 14.5 Mb	3A, 3D, 3H, 5X	Yu et al. 2021
B. rapa (Chineese cabbage)	*PbBa8.1*	QTL	…	Pb4	Chen et. al. 2013
B. rapa cv ECD01	*Bra.CR.b,*	QTL	..	..	Hirani et al. 2018
B. rapa cv ECD02	*Rcr9^{ECD02}*	QTL	10.7 – 12.2 Mb	3A, 3D, 3H, 5X	Rahaman et al. 2022
B. napus cv. AAFC695	*Rcr9^{AAFC695}*	QTL	10.8 – 11.6 Mb	3A, 3D, 3H, 5X	Rahaman et al. 2022
B. napus cv Mendel	*Rcr3^{Mendel}*	QTL	11.0 – 12.3 Mb	3D, 3H, 5C, 5J	Rahaman et al. 2022
B. napus L. subsp. *rapifera* Metzg.	Major QTL	QTL	9.96 –11.09 Mb	3A, 3D, 3H	Wang et al. 2022

**Physical position has been determined with *B. rapa* v3.0 (cv. Chiifu)

5.7. Figures

Figure 5. 1. Clubroot disease development on the clubroot-resistant *Brassica napus* cv. 'Mendel' and F_1 of DH16516 × 'Mendel' shown in comparison with the susceptible parent DH16516 ('DHT') used in the study.

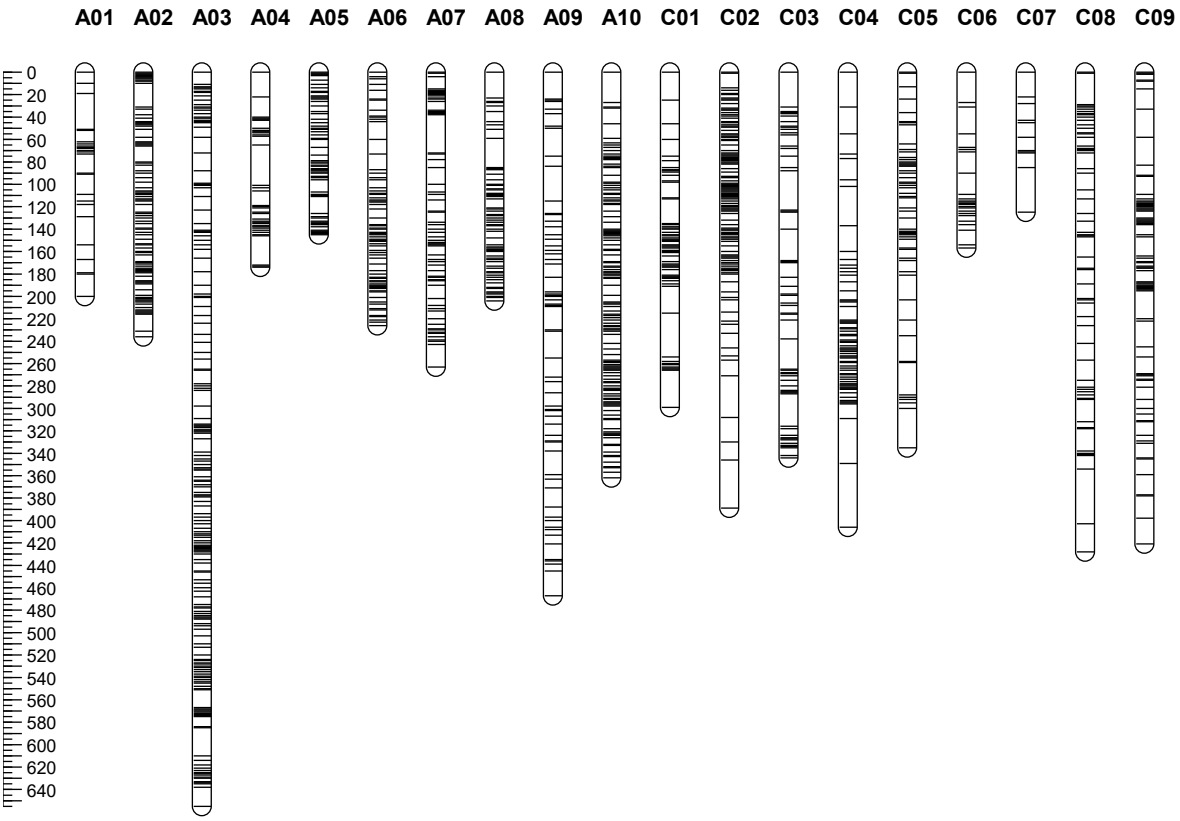


Figure 5.2**.** Genetic linkage map of 137 doubled haploid (DH) *Brassica napus* lines of a population derived from DH16516 × 'Mendel'. The linkage map consists of 2,642 high-quality SNP markers distributed to the *B. napus* genome profile of 19 chromosomes. The vertical scale on the left indicates the genetic distance in centiMorgan (cM).

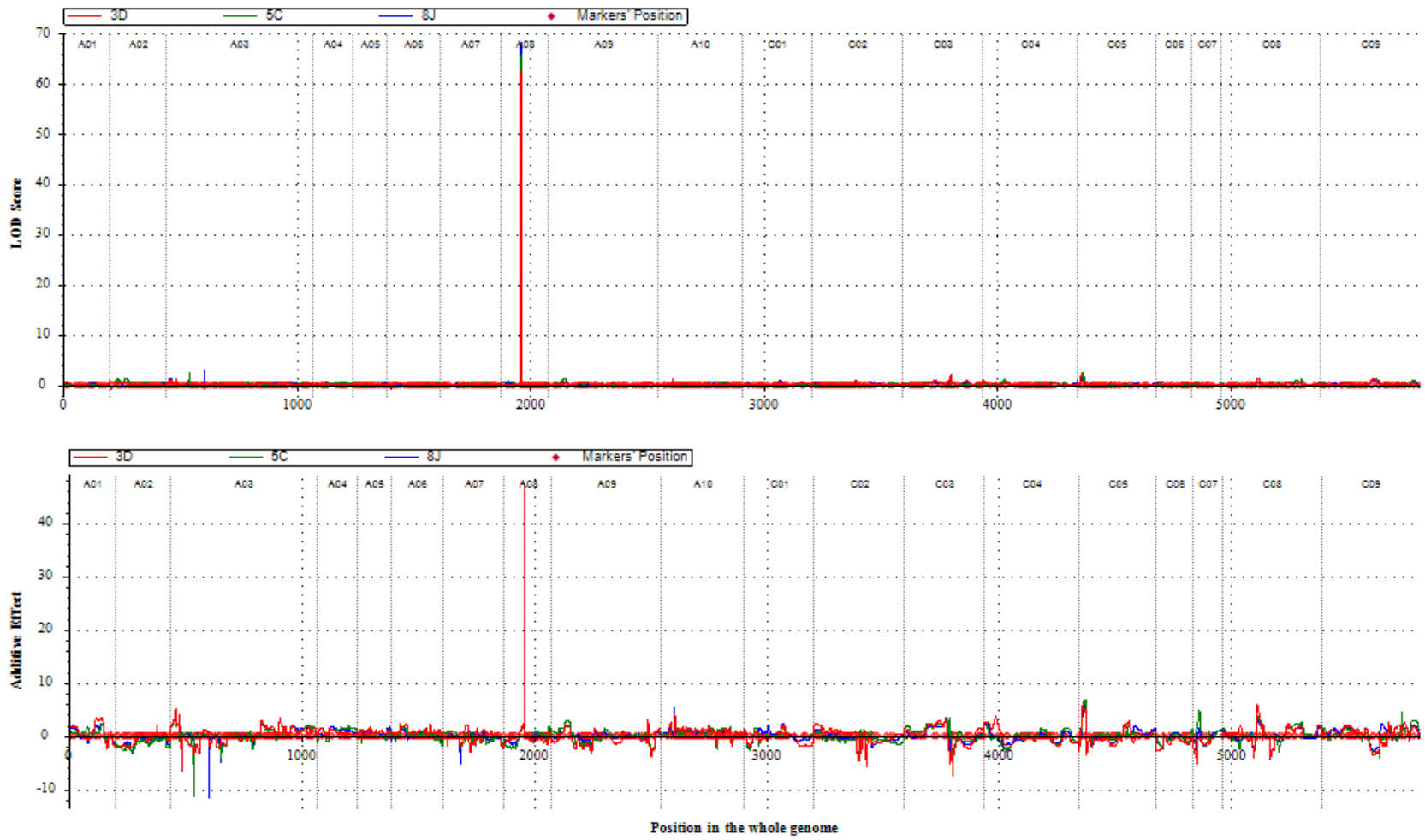


Figure 5. 3**.** The effect of *Rcr3*^{Mendel} on the phenotypic expression of resistance to pathotypes 3D, 5C, and 8J of *Plasmodiophora brassicae*, based on LOD value and additive effect of the identified QTL considering the whole genome of *Brassica napus* 'Mendel'.

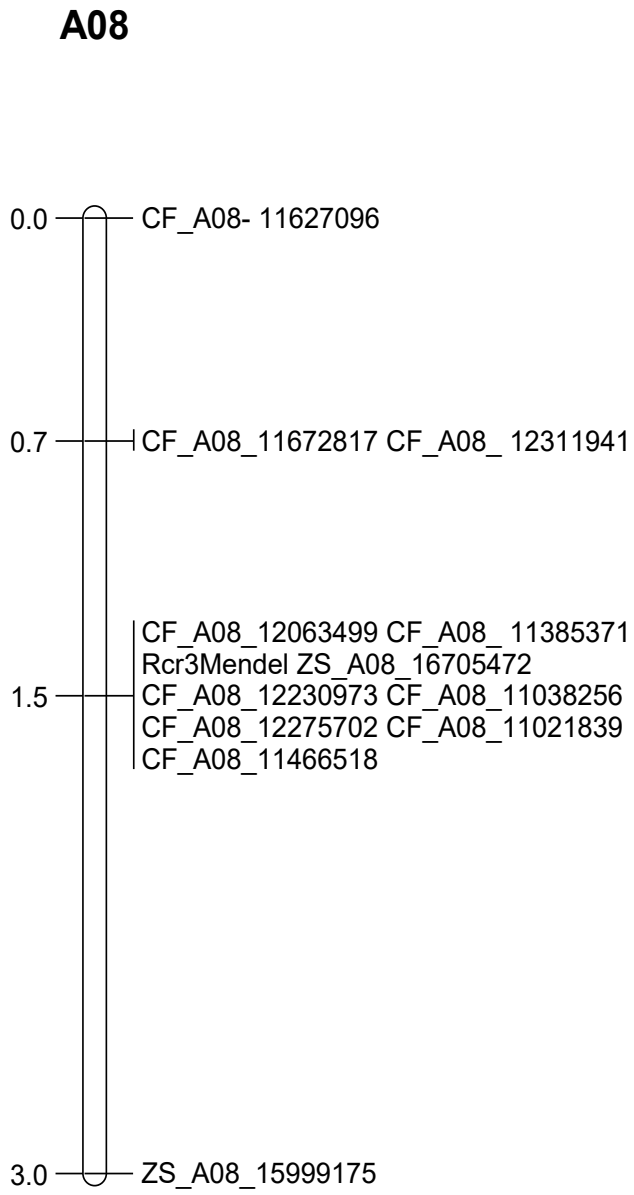


Figure 5. 4**.** The position of the identified QTL/gene *Rcr3*^{Mendel} on chromosome A08 is illustrated by the linkage map drawn using 10 SNPs from a KASP assay and 4 SNPs identified by QTL analysis in the current study.

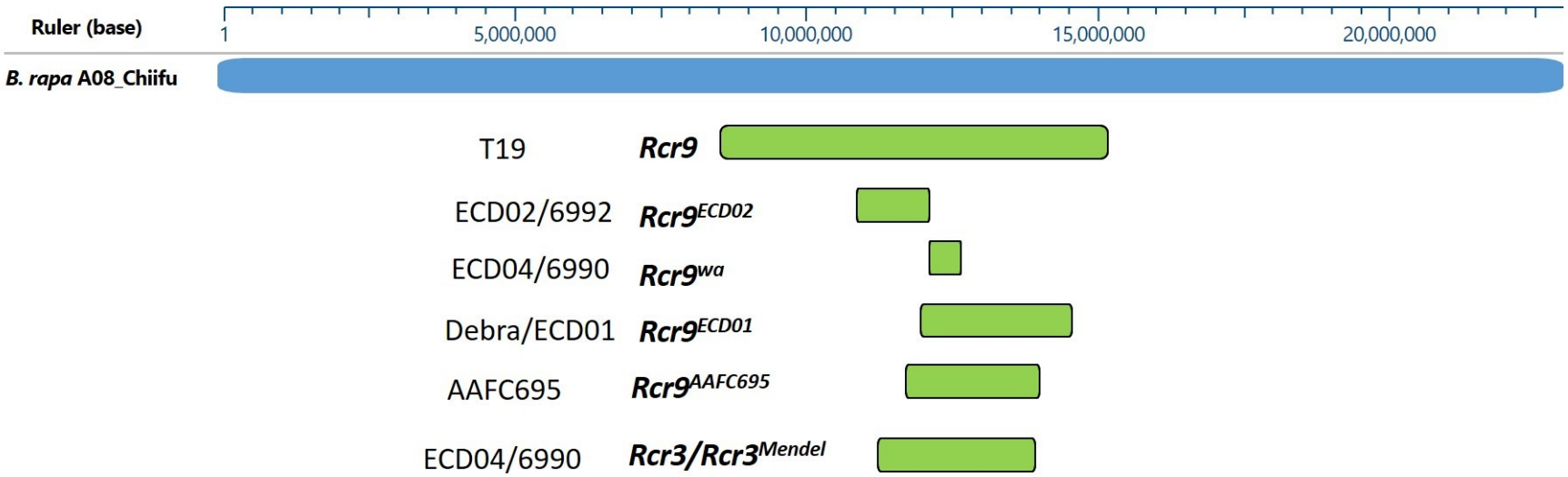


Figure 5. 5. Corresponding location of the identified QTL *Rcr3*/*Rcr3*^{Mendel} on chromosome A08 of the *Brassica rapa* genome v3.0 (Chiifu) in relation to the previously identified QTL *Rcr9* (Yu et al. 2017), *Rcr9*^{ECD02} (Rahaman et al. 2022), *Rcr9*^{AAFC695} (Chapter 4), *Rcr9*^{wa} (Karim et al. 2020), and *Rcr9*^{ECD01} (Yu et al. 2021) from different populations.

Chapter 6. General discussion

Brassica species are one of the largest eudicot groups in the Brassicaceae family, consisting of around 338 genera and 3709 species (Warwick et al. 2006; Cheng et al. 2014). The leaves, buds, roots, stems, and seeds of the *Brassicas* are used as oilseed, forage, condiments, and vegetables. Studies indicate that three amphidiploid species developed naturally from wild diploid species (U 1935) through natural hybridization and doubling of diploid chromosomes. For example, *B. napus,* which carries the genomes AACC, 2n = 38, developed from interspecific hybridization between *B. rapa* (AA, 2n = 20) and *B. oleracea* (CC, 2n = 18) (Nagaharu 1935; Raymer 2002). *Brassica napus* canola (≡ oilseed rape) supplies 13% to 16% of the global market for edible oil (Song et al. 2020), second only to soybean (Kupiec et al. 2020; USDA, 2020). In Canada, canola is one of the most important cash crops, contributing $29.9 billion annually to the national economy (Rempel et al. 2014; Botero et al. 2019; Canola Council of Canada 2020).

Clubroot, caused by *Plasmodiophora brassicae*, has become an important constraint to canola production in Canada (Strelkov & Hwang 2014; Strelkov et al. 2016). The resting spores of the pathogen can survive in infested soil for many years (Braselton 1995; Wallenhammar 1996; Peng et al. 2015) and can cause yield losses of up to 30–100% (Tewari et al. 2005; Strelkov et al. 2007). More than 36 pathotypes of *P. brassicae* have been identified from Canada (Strelkov et al. 2018; Hollman et al. 2021), and resistance to many of these pathotypes is not available in commercial canola cultivars (Strelkov et al. 2016; Strelkov et al. 2018; Hollman et al. 2021). In addition to its impact on the Canadian canola industry, clubroot is also an important disease of *B. napus* and/or other cruciferous crops in Australia, China, Japan, Germany, France, the Czech Republic, Indonesia, Thailand, Vietnam, Sweden, Britain, Latin America, and the USA (Donald 2005; Donald and Porter 2014; Tanaka et al. 2001; Diederichsen et al. 2014;

Wallenhammar 1996; Botero-Ramirez et al. 2019; Chittem et al. 2014). The focus of this thesis was on the identification and mapping of clubroot resistance genes effective against some of the most important resistance-breaking pathotypes of *P. brassicae* in Canada.

The clubroot response of lines of *B. rapa* and *B. napus* to pathotypes 2B, 3A, and 3D were assessed, and one of the best performing lines, 'AAFC695', was selected for detailed analysis. A segregating population from 'AAFC695' was developed for use in genetic mapping. Similar studies were conducted with two other clubroot-resistant (CR) genotypes, ECD02 and 'Mendel'. Among the 50 *B. napus* and *B. rapa* lines tested, the *B. napus* lines 'AAFC695' and CGN06874 were found to be highly resistant. In contrast, none of the *B. rapa* lines were resistant to the pathotypes assessed.

Doubled haploid (DH) lines were developed via microspore culture and doubling chromosomes. This approach allowed for gene mapping in a population descended from a wild progenitor in a single generation (Cao et al. 2016). It also allowed for the assessment of the reaction to several pathotypes in a replicated trial. There is a single cycle of meiosis in DH populations, which results in fewer crossover and larger chromosomal blocks in the genome relative to BC populations. Moreover, *B. napus* species have a complex amphidiploid genome profile, so mapping genes from a DH population is less difficult relative to a segregating population. Therefore, the DH population was selected to map genes in *B. napus* populations, but a BC population was used for the diploid species *B. rapa*.

Genotype-by-Sequencing (GBS) was used to identify one major QTL, $Rcr9^{ECD02}$, on the A08 chromosome of *B. rapa* line ECD02; this QTL conferred resistance to four pathotypes of *P. brassicae,* 3A, 3D, 3H, and 5X. The results were confirmed by BSA and KASP genotyping. The QTL was tagged by SNP markers; on the left, marker CF_A08_12331263 (399.5 cM) and on the

right, marker CF_A08_10575267 (402.5). The QTL explained 68.9 to 77.4% of PVE to the individual pathotypes, with corresponding LOD values of 24.3 to 31.1 with a type I error rate of 0.01, and additive values of 64.2 to 74.7%. This QTL differed from several CR loci recently identified on A08 of *B. rapa* (Yu et al. 2017; Karim et al. 2020; Yu et al. 2021; Hirani et al. 2018). However, it could not be assessed against the position of the QTL/genes *Crr1*, *CRs*, *PbBa8.1*, and *qBrCR38-2* (Suwabe et al. 2003, Laila et al. 2019; Chen et al. 2013; Zhu et al. 2019), because no information regarding the corresponding region for these genes on the *B. rapa* 'Chiifu' reference genome v3.0 is available.

Two other QTL, $Rcr9^{AAFC695}$ *and* $Rcr3^{Mendel}$, were identified from the *B. napus* cultivars 'AAFC695' and 'Mendel'. $Rcr9^{AAFC695}$ explained 92.6% of the phenotypic variation for reaction to inoculation with pathotype 3A, with an additive effect of 47.2% and a LOD value of 60.3. This QTL also explained 95.0%, 94.9%, and 93.4% of the resistance to pathotypes 3D, 3H, and 5X, respectively, with an additive effect of 48.7%, 48.7% and 47.7%, and corresponding LOD values of 70.8, 70.4, and 63.3. The identified QTL spanned between 76.74 cM and 83.44 cM on the linkage group A08.

The QTL $Rcr9^{Mendel}$ explained 88% of the phenotypic variation for the reaction to pathotype 3D, with an additive effect of 47% and LOD value 63. It also explained 89% of the phenotypic variation in response to pathotype 5C and 90% of the variation to pathotype 8J, with an additive effect of 47% for both pathotypes and LOD values of 66 and 68, respectively. The reaction of a subset of the population to pathotype 3H was used to determine if the $Rcr3^{Mendel}$ region was homologous to the *Rcr3* identified previously. The QTL *Rcr3*/$Rcr3^{Mendel}$ explained 33.2% PVE with a corresponding LOD value 5.3, and the nearest marker, ZS_A08_15999175, was the same as the marker identified to tag $Rcr3^{Mendel}$ for pathotypes 3D, 5C and 8J. KASP

analysis to identify closely linked markers indicated that *Rcr9*^{AAFC695} co-segregated with several SNP markers: CF_A08_ 11385371, CF_A08- 11627096, CF_A08_10850975, CF_A08_10855744, CF_A08_11038256, CF_A08_11059924, CF_A08_11219809, CF_A08_11672817, and CF_A08_11855997. It also showed that *Rcr3*^{Mendel} co-segregated with ZS_A08_16705472, CF_A08_11385371, CF_A08_12063499, CF_A08_12230973, CF_A08_11038256, CF_A08_12275702, CF_A08_11021839, and CF_A08_11466518. These results suggested that the *Rcr3* and *Rcr3*^{Mendel} regions are homologous.

The *Rcr3*^{Mendel} region was shown to be homologous to the genomic region of *B. rapa* v3.0 where *Rcr9*^{ECD02} and *Rcr9*^{AAFC695} have been identified (Chapters 3 and 4). However, it was concluded that *Rcr9*^{ECD02} and *Rcr9*^{AAFC695} are different from *Rcr3*^{Mendel} based on the resistance reactions to several pathotypes; *Rcr9*^{ECD02} conferred resistance to pathotypes 3A, 3D, 3H, and 5X, but lines carrying *Rcr3*^{Mendel} were susceptible to 3A and 5X. Therefore, *Rcr3*^{Mendel} is not the same as *Rcr9*^{ECD02}. Similarly, *Rcr3*^{Mendel} and *Rcr9*^{ECD02} are different from *Rcr9*^{AAFC695}; while *Rcr9*^{ECD02} conferred resistance to the same pathotypes (3A, 3D, 3H, and 5X) used to map resistance genes in the *B. napus* 'AAFC695', the TIR-NB-LRR (TNL) genes identified in *Rcr9*^{ECD02} and *Rcr9*^{AAFC695} were different. Two TNL genes, *BnaA08T0141000ZS* and *BnaA08T0142200ZS*, were identified in the region of *Rcr9*^{AAFC695} (homologous to *BraA08g017130.3C* and *BraA08g016670.3C* in *B. rapa* Chiifu v3.0), but four other disease resistance genes were identified in the region of *Rcr9*^{ECD02}. Although the QTL/genes *Rcr9*^{ECD02}, *Rcr9*^{AAFC695} and *Rcr3*^{Mendel} were all mapped to the A08 chromosome of *B. rapa* and *B. napus*, it was difficult to separate the genes based on location. This supports the conclusion from previous studies that there is a cluster of genes on chromosome A08 associated with clubroot resistance

(Suwabe et al. 2003, 2006; Hatakeyama et al. 2013; Laila et al. 2019; Zhu et al. 2019; Karim et al. 2020; Hasan & Rahman 2016).

Most CR canola cultivars are believed to carry a single major gene for clubroot resistance. Previous studies have indicated that single-gene resistance can quickly be eroded by novel pathotypes of *P. brassicae* (Fredua-Agyeman et al. 2018; LeBoldus et al. 2012; Peng et al. 2014; Rahman et al. 2014; Strelkov et al. 2016, 2018). Pyramiding multiple clubroot resistance genes in a single host genotype (Matsumoto et al. 2012; Tomita et al. 2013) may provide more durable resistance against *P. brassicae*. Using high-throughput NGS technology in combination with BSA and KASP analysis, the current study identified several novel QTL/genes with strong efficacy against some important new pathotypes. These genes could be used in combination to develop more durable CR canola cultivars.

The rapid emergence of new pathotypes of *P. brassicae* in Canada since 2013 represents a threat to the successful production of canola. To help manage this changing pathogen population, several new QTL/genes for resistance were identified that were effective against resistance-breaking pathotypes of *P. brassicae*. All of the QTL/genes were located on the A08 chromosome, where a cluster of genes conferring clubroot resistance has been reported previously. The novel resistance genes identified in this study represent an important resource, as they could be introgressed and combined in commercial canola cultivars for improved clubroot resistance. Fine mapping and cloning of these genes should also be carried out in the future, to determine whether it is the cluster of genes or specific genes that are responsible for the clubroot resistance. Ultimately, an integrated approach, combining different resistance genes with other clubroot management strategies, will be required for the sustainable management of this disease.

Conclusions

The research presented in this thesis:

I. Identified *B. napus* genotypes resistant to new resistance-breaking pathotypes of *P. brassicae* identified in Canada

II. Developed a backcross population from the clubroot resistant *B. rapa* line ECD02 crossed with a highly susceptible *B. rapa* DH line ACDC [ACDC × (ACDC × ECD02)], mapped a major QTL conferring resistance to pathotypes 3A, 3D, 3H and 5X, and identified six markers tightly linked to and co-segregating with the QTL

III. Developed a *B. napus* DH population through microspore culture from the F_1 of DH16516 × AAFC695, identified and mapped a major QTL on the A08 chromosome for resistance to pathotypes 3A, 3D, 3H, and 5X, identified markers tightly linked to and co-segregating with the QTL, and identified and mapped the QTL position in *B. napus* corresponding to the region in the *B. rapa* genome v3.0 (Chiifu)

IV. Developed a *B. napus* DH population through microspore culture from the F_1 of DH16516 × Mendel, mapped a new QTL on chromosome A8 conferring resistance to pathotypes 3D, 5C, and 8J, identified eight co-segregated and tightly linked markers with the QTL, and determined the QTL position in *B. napus* corresponding to the region in the *B. rapa* genome v3.0 (Chiifu)

Future research

A number of potential new studies could stem from the research presented in this thesis, which could help to advance our knowledge further. These include:

I. Fine mapping of the genes in the identified QTL with a large number of populations

II. Cloning of the resistance genes in the identified QTL to determine whether the cluster of genes or a specific gene is responsible for conferring resistance

III. Introgression and stacking of the resistance gene(s) in the identified QTL into commercial canola cultivars for more durable clubroot resistance

IV. Use of developed SNP markers for MAS that can be genotyped through KASP technology

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Appendix

Supplemental Tables

Table S2. 1. Reaction of 16 *Brassica rapa* families to the resistance-breaking pathotypes 2B, 3A, and 3D of *Plasmodiophora brassicae* identified in Alberta, Canada.

Genotype/ line/ cultivar	Species	Type	Pathotype/disease reaction					
			2B		3A		3D	
			# Plants	Reaction	# Plants	Reaction	# Plants	Reaction
T19-X17	*B. rapa*	BC1S1	30	S	33	S	36	S
T19-X27	*B. rapa*	BC1S1	33	S	36	S	36	S
T19-X28	*B. rapa*	BC1S1	34	S	36	S	36	S
T19-X33	*B. rapa*	BC1S1	32	S	33	S	35	S
T19-X38	*B. rapa*	BC1S1	29	S	34	S	34	S
T19-X46	*B. rapa*	BC1S1	32	S	35	S	34	S
T19-X59	*B. rapa*	BC1S1	33	S	36	S	32	S
T19-X65	*B. rapa*	BC1S1	31	S	36	S	33	S
T19-X66	*B. rapa*	BC1S1	33	S	36	S	36	S
T19-X71	*B. rapa*	BC1S1	33	S	32	S	36	S
T19-X78	*B. rapa*	BC1S1	36	S	35	S	36	S
T19-X79	*B. rapa*	BC1S1	36	S	34	S	36	S
T19-X80	*B. rapa*	BC1S1	34	S	36	S	36	S
T19-X82	*B. rapa*	BC1S1	32	S	36	S	36	S
T19-X84	*B. rapa*	BC1S1	33	S	36	S	36	S
T19-X92	*B. rapa*	BC1S1	36	S	35	S	36	S
ECD-05 (C)	*B. rapa*	Cultivr	35	S	36	S	36	S

*(C) indicates the check/control; R, resistance/S, susceptible
indicates- 'the number of'

Table S2. 2**.** Reaction of 34 *Brassica napus* accessions to the resistance-breaking pathotypes 2B, 3A, and 3D of *Plasmodiophora brassicae* identified in Alberta, Canada.

Genotype/ line/ cultivar	Habit	Type	Origin	Pathotype/disease reaction					
				2B		3A		3D	
				# Plants	Rc.	# Plants	Rc.	# Plants	Rc.
ECD06	Winter	Oilseed rape	Europe	36	S	33	S	36	S
ECD09	Winter	Oilseed rape	Europe	30	S	36	S	36	S
DH6756-5	Spring	Oilseed rape	Canada	36	S	36	S	36	S
CN31153	Winter	Rutabaga	Europe	36	S	36	S	36	S
CN31154	Winter	Rutabaga	Europe	32	S	36	S	36	S
CN31304	Winter	Rutabaga	Europe	36	S	36	S	36	S
CN31391	Winter	Rutabaga	Europe	36	S	36	R	35	S
CN31403	Winter	Rutabaga	Europe	32	S	36	S	36	S
CN31417	Winter	Rutabaga	Europe	33	S	36	S	36	S
CN31450	Winter	Rutabaga	Europe	24	S	24	S	28	S
CN31451	Winter	Rutabaga	Europe	36	S	36	S	36	S
CN31452	Winter	Rutabaga	Europe	36	S	35	S	36	S
CN31454	Winter	Rutabaga	Europe	32	S	36	S	36	S
CN31457	Winter	Rutabaga	Europe	36	S	31	R	32	S
CN35993	Winter	Oilseed rape	Asia	36	S	33	S	36	S
CN39440	Winter	Oilseed rape	Asia	36	S	36	S	35	S
CN39441	Winter	Oilseed rape	Asia	36	S	36	S	36	S
CN39443	Winter	Oilseed rape	Asia	36	S	35	S	36	S
CN43206	Spring	Oilseed rape	Asia	36	S	34	S	35	S
CN46235	Spring	Oilseed rape	North America	36	R	36	R	36	R
Ames1669	Spring	Oilseed rape	North America	14	S	16	S	13	S
Ames6075	Spring	Oilseed rape	North America	36	S	35	S	36	S

Genotype/ line/ cultivar	Habit	Type	Origin	Pathotype/disease reaction					
				2B		3A		3D	
				# Plants	Rc.	# Plants	Rc.	# Plants	Rc.
PI 284859	Winter	Oilseed rape	Europe	35	S	36	R	36	R
PI 305280	Winter	Oilseed rape	Europe	36	S	35	S	36	S
PI 311727	Winter	Oilseed rape	Europe	32	S	36	S	35	S
CGN06822	Winter	Oilseed rape	Europe	12	S	11	S	12	S
CGN06902	Winter	Oilseed rape	Europe	20	R	23	R	24	R
CGN07237	Winter	Oilseed rape	Europe	36	S	36	S	32	S
CGN06896	Winter	Oilseed rape	Europe	32	S	36	S	36	S
CGN17369	Winter	Oilseed rape	Europe	36	R	36	R	35	R
AAFC695	Winter	Oilseed rape	Oceania	36	R	36	R	34	R
CGN17381	Winter	Oilseed rape	Europe	36	S	35	S	36	S
CGN13919	Winter	Oilseed rape	Europe	36	S	36	S	36	S
Laurentian	Winter	Rutabaga	Europe	36	S	36	S	36	S
DH 16516	Spring	Oilseed rape	North America	36	S	35	S	36	S
Westar (C)	Spring	Oilseed rape	Europe	35	S	36	S	36	S

*(C) indicates the check/control; R, resistant/S, susceptible
indicates- 'the number of'
Rc, Reaction to the host

Table S3. 1. Segregation characteristics of the *Brassica rapa* parental lines ACDC and ECD02, BC_1S_1 population derived from ACDC × (ACDC × ECD02), and susceptible and resistant checks used in this study.

Lines/ crosses	Pathotypes/ DSI							
	3A		3D		3H		5X	
	R	S	R	S	R	S	R	S
ECD02 (P2)	12	0	12	0	12	0	12	0
ACDC (P1)	0	12	0	12	0	12	0	12
DHT (DH S-Line)	0	29	0	25	0	139	0	19
NRC11-24 (DH S-line)	0	88	0	136	-	-	0	110
Y12 (DH R-line)	28	0	28	0	18	0	26	0
Y68 (DH R-line)	37	0	30	0	36	0	32	0
$\chi 2$	-	-	-	-	-	-	-	-
P-value	-	-	-	-	-	-	-	-
$ECD02BC_1S_1$	45	48	45	48	44	49	43	50
$\chi 2$	0.093		0.093		0.258		0.505	
P-value	0.761		0.761		0.612		0.478	

**DSI<60 = R (resistant); DSI≥60 =S (susceptible)

Table S3. 2**.** Statistics of the *Brassica rapa* ECD02 BC_1S_1 population, derived from ACDC × (ACDC × ECD02), tested for resistance to the *Plasmodiophora brassicae* pathotypes 3A, 3D, 3H, and 5X.

Patho-type	Sample Size	Min.	Max.	Range	Mean	Vari-ance	Std. Error	Skew-ness	Kurt-osis	W-test
3A	93	0.00	100	100	59.4	1768.3	42.1	-0.23	-1.71	0.75
3D	93	0.00	100	100	59.4	1799.4	42.4	-0.21	-1.77	0.73
3H	93	0.00	100	100	59.7	1847.8	43.0	-0.29	-1.69	0.73
5X	93	0.00	100	100	64.6	1494.2	38.7	-0.39	-1.49	0.76

Table S3. 3**.** Number of missing bases per SNP markers in the ECD02 BC_1S_1 population derived from ACDC × (ACDC × ECD02).

Chrom.	Total SNPs	Total 'a'	Total 'h'	# Missing bases	Missing (%)	Missing range
A01	136	7429	5744	19	0.14	1-11
A02	130	6152	6429	29	0.23	1-2
A03	159	9510	5872	41	0.27	1-12
A04	115	6428	4702	25	0.22	1-2
A05	146	8936	5187	39	0.28	1-11
A06	141	8511	5104	62	0.46	1-10
A07	131	7932	4697	78	0.62	1-9
A08	135	7651	5432	12	0.09	1-2
A09	105	5801	4349	35	0.34	1-3
A10	146	7840	6313	9	0.06	0-1
Total	1344	76190	53829	349	2.72	-

Table S3. 4. Disease Severity Index (DSI) of the ECD02BC_1S_1 lines, derived from ACDC × (ACDC × ECD02), following inoculation with the *Plasmodiophora brassicae* pathotypes 3A, 3D, 3H, and 5X.

	DSI			
Lines/ Pathotypes	3A	3D	3H	5X
ECD02-01	0.0	16.7	11.1	20.0
ECD02-02	22.2	10.0	37.5	16.7
ECD02-03	100.0	100.0	100.0	100.0
ECD02-04	0.0	37.5	16.7	0.0
ECD02-05	25.0	20.0	20.0	28.6
ECD02-06	100.0	100.0	100.0	100.0
ECD02-07	14.3	37.5	0.0	50.0
ECD02-08	0.0	20.0	14.3	33.3
ECD02-09	33.3	14.3	25.0	50.0
ECD02-10	0.0	16.7	0.0	16.7
ECD02-11	100.0	100.0	100.0	100.0
ECD02-12	100.0	100.0	100.0	100.0
ECD02-13	100.0	100.0	100.0	100.0
ECD02-14	60.0	42.9	66.7	20.0
ECD02-15	100.0	100.0	100.0	100.0
ECD02-16	100.0	100.0	100.0	100.0
ECD02-17	100.0	100.0	100.0	100.0
ECD02-18	100.0	100.0	100.0	100.0
ECD02-19	100.0	100.0	100.0	100.0

	DSI			
Lines/ Pathotypes	3A	3D	3H	5X
ECD02-20	100.0	100.0	100.0	100.0
ECD02-21	100.0	100.0	100.0	100.0
ECD02-22	100.0	100.0	100.0	100.0
ECD02-23	42.9	20.0	14.3	33.3
ECD02-24	100.0	100.0	100.0	100.0
ECD02-25	100.0	100.0	100.0	100.0
ECD02-26	33.3	20.0	33.3	33.3
ECD02-27	11.1	50.0	0.0	45.5
ECD02-28	33.3	25.0	28.6	25.0
ECD02-29	25.0	16.7	0.0	27.8
ECD02-31	40.0	0.0	33.3	33.3
ECD02-32	0.0	0.0	0.0	50.0
ECD02-33	100.0	100.0	100.0	100.0
ECD02-34	0.0	20.0	0.0	50.0
ECD02-35	100.0	100.0	100.0	100.0
ECD02-36	25.0	28.6	25.0	22.2
ECD02-37	100.0	100.0	100.0	100.0
ECD02-38	42.9	25.0	20.0	25.9
ECD02-39	12.5	0.0	25.0	37.5
ECD02-40	14.3	16.7	9.5	0.0
ECD02-41	33.3	50.0	0.0	20.0

	DSI			
Lines/ Pathotypes	3A	3D	3H	5X
ECD02-42	100.0	100.0	100.0	100.0
ECD02-43	100.0	100.0	100.0	100.0
ECD02-44	33.3	9.1	20.8	33.3
ECD02-45	100.0	100.0	100.0	100.0
ECD02-46	100.0	100.0	100.0	100.0
ECD02-47	100.0	85.7	100.0	100.0
ECD02-48	33.3	28.6	50.0	20.0
ECD02-49	100.0	100.0	100.0	100.0
ECD02-51	100.0	100.0	100.0	100.0
ECD02-52	16.7	16.7	0.0	28.6
ECD02-57	100.0	87.5	100.0	100.0
ECD02-59	33.3	14.3	0.0	33.3
ECD02-63	25.0	0.0	0.0	50.0
ECD02-64	100.0	100.0	70.0	100.0
ECD02-65	83.3	100.0	100.0	100.0
ECD02-66	100.0	100.0	100.0	100.0
ECD02-67	100.0	100.0	100.0	100.0
ECD02-68	12.5	20.0	50.0	25.0
ECD02-69	10.0	0.0	28.6	42.9
ECD02-70	66.7	100.0	75.0	100.0
ECD02-71	100.0	100.0	100.0	100.0

	DSI			
Lines/ Pathotypes	3A	3D	3H	5X
ECD02-72	8.3	26.7	0.0	50.0
ECD02-74	0.0	0.0	0.0	0.0
ECD02-75	0.0	0.0	25.0	0.0
ECD02-76	100.0	100.0	100.0	100.0
ECD02-77	0.0	0.0	0.0	33.3
ECD02-78	100.0	100.0	100.0	75.0
ECD02-79	33.3	0.0	0.0	50.0
ECD02-80	0.0	0.0	33.3	0.0
ECD02-81	100.0	100.0	100.0	100.0
ECD02-82	40.0	50.0	50.0	40.0
ECD02-84	100.0	100.0	100.0	80.0
ECD02-85	50.0	0.0	50.0	0.0
ECD02-86	0.0	33.3	50.0	85.7
ECD02-87	0.0	33.3	0.0	30.0
ECD02-88	0.0	0.0	0.0	0.0
ECD02-89	0.0	33.3	0.0	0.0
ECD02-91	20.0	14.3	14.3	80.0
ECD02-92	100.0	100.0	100.0	100.0
ECD02-93	33.3	0.0	42.9	0.0
ECD02-94	100.0	100.0	100.0	100.0
ECD02-95	100.0	100.0	100.0	100.0

	DSI			
Lines/ Pathotypes	3A	3D	3H	5X
ECD02-97	100.0	100.0	100.0	100.0
ECD02-98	100.0	100.0	100.0	100.0
ECD02-99	100.0	100.0	100.0	100.0
ECD02-100	100.0	100.0	100.0	100.0
ECD02-101	100.0	100.0	100.0	100.0
ECD02-103	100.0	77.8	100.0	100.0
ECD02-105	100.0	100.0	100.0	100.0
ECD02-106	79.2	100.0	100.0	100.0
ECD02-107	100.0	100.0	100.0	100.0
ECD02-108	100.0	100.0	100.0	100.0
ECD02-109	75.0	100.0	100.0	100.0

Table S4. 1. Information on the *Brassica napus* reference genome ZS11 (Song et al. 2020) used to map CR genes in *Brassica napus* cultivar 'AAFC695'.

Reference genome	Assembly size (Mb)	Anchored assemblies to the chrom. (Mb)	Number of scaffold N50 (Mb)	Total TEs (%)	Number of annotated genes	Completeness (%, CEGMA)
ZS11	1,008	961 (95.3%)	57.88	55.62	100,919	99.19

*CEGMA: a software pipeline used to annotate the core genes in eukaryotic genomes.

Table S4. 2**.** Phenotypic distribution of 102 doubled haploid (DH) lines, derived from the F_1 of DH16516 × AAFC695, tested against *Plasmodiophora brassicae* pathotypes 3A, 3D, 3H, and 5X.

Trait Name	Sample Size	Mean	Variance	Std. Error	Skewness	Kurtosis	S-Wilk test	Min.	Max.
3A	102	48.1	2400.2	49	0.1	-2	0.643	0	100
3D	102	49.7	2497.1	50	0	-2	0.621	0	100
3H	102	49.6	2493.7	49.9	0	-2	0.622	0	100
5X	102	51.1	2435.3	49.3	0	-2	0.636	0	100

Table S4. 3**.** Assembly summaries of the parental lines DH16516 and 'AAFC695', and a *Brassica napus* 'AAFC695' doubled haploid (DH) population derived from the F_1 of DH16516 × AAFC695.

Parents/ population	Total sequences	Assembled reads	% Assembly	Total number of SNPs	SNPs/ 1000 assembled reads
DH16516 (Parent-1)	219.9×10^6	172.8×10^6	78.6	9.02 M	52.2
AAFC695 (Parent-2)	7.42×10^6	6.22×10^6	83.8	0.024 M	39.01
AAFC695 DH (102 Lines)	520.7×10^6	404.7×10^6	77.7	2.12 M	52.02

Table S4. 4**.** Marker distribution of the high density genetic linkage map of a *Brassica napus* 'AAFC695' doubled haploid (DH) population derived from the F_1 of DH16516 × AAFC695.

Chromosomes		Number of Markers	Length (cM)	Minimum Interval (cM)	Maximum Interval (cM)	Average Interval (cM)
ZS_A01		12	53.38	0	13.77	4.45
ZS_A02		106	106.22	0	11.18	1
ZS_A03		236	219.51	0	11.07	0.93
ZS_A04		135	100.88	0	5.64	0.74
ZS_A05		115	130.69	0	18.27	1.14
ZS_A06		105	106.69	0	6.9	1.02
ZS_A07		125	74.54	0	4.18	0.6
ZS_A08		97	87.37	0	6.22	0.9
ZS_A09		248	213.56	0	8.07	0.86
ZS_A10		141	108.47	0	7.05	0.77
ZS_C01		5	39.36	0	23.04	7.87
ZS_C02		232	87.52	0	12.44	0.38
ZS_C03		185	177.45	0	20	0.96
ZS_C04		25	43.21	0	5.39	1.73
ZS_C05		138	127.69	0	22.19	0.93
ZS_C06		90	47.14	0	5.22	0.52
ZS_C07		174	178.3	0	24.17	1.02
ZS_C08		122	129.4	0	13.58	1.06
ZS_C09		89	107.39	0	18.73	1.21
Whole-genome	Total	2380	2138.77			
	Mean	125	112.57	0	12.48	1.48

Table S4. 5. Disease Severity Index (DSI) of 102 'AAFC695' doubled haploid lines, derived from the F_1 of DH16516 × AAFC695, following inoculation with *Plasmodiophora brassicae* pathotypes 3A, 3D, 3H, and 5X.

	DSI			
Lines/ pathotypes	3A	3D	3H	5X
AAFC695DH-01	100.0	100.0	100.0	100.0
AAFC695DH-02	100.0	100.0	100.0	100.0
AAFC695DH-03	100.0	100.0	100.0	100.0
AAFC695DH-04	0.0	0.0	0.0	0.0
AAFC695DH-05	0.0	0.0	0.0	0.0
AAFC695DH-06	90.9	100.0	100.0	100.0
AAFC695DH-07	0.0	0.0	0.0	0.0
AAFC695DH-08	0.0	0.0	0.0	0.0
AAFC695DH-09	100.0	100.0	100.0	100.0
AAFC695DH-10	100.0	100.0	100.0	100.0
AAFC695DH-11	62.5	100.0	100.0	100.0
AAFC695DH-12	0.0	0.0	0.0	0.0
AAFC695DH-13	100.0	100.0	100.0	100.0
AAFC695DH-14	100.0	100.0	100.0	100.0
AAFC695DH-15	100.0	100.0	100.0	100.0
AAFC695DH-16	0.0	0.0	0.0	0.0
AAFC695DH-17	0.0	0.0	0.0	0.0
AAFC695DH-18	0.0	0.0	0.0	0.0
AAFC695DH-19	100.0	100.0	100.0	100.0

	DSI			
Lines/ Pathotypes	3A	3D	3H	5X
AAFC695DH-20	100.0	100.0	100.0	100.0
AAFC695DH-21	77.8	100.0	100.0	100.0
AAFC695DH-22	0.0	0.0	0.0	0.0
AAFC695DH-23	0.0	0.0	0.0	0.0
AAFC695DH-24	0.0	0.0	0.0	0.0
AAFC695DH-25	77.8	100.0	100.0	100.0
AAFC695DH-26	36.4	100.0	100.0	100.0
AAFC695DH-27	0.0	0.0	0.0	0.0
AAFC695DH-28	100.0	100.0	100.0	100.0
AAFC695DH-29	0.0	0.0	0.0	25.0
AAFC695DH-30	100.0	100.0	100.0	100.0
AAFC695DH-31	100.0	100.0	100.0	90.9
AAFC695DH-32	0.0	0.0	0.0	0.0
AAFC695DH-33	100.0	100.0	100.0	100.0
AAFC695DH-34	100.0	100.0	77.8	100.0
AAFC695DH-35	100.0	100.0	100.0	100.0
AAFC695DH-36	0.0	0.0	0.0	0.0
AAFC695DH-37	0.0	0.0	0.0	0.0
AAFC695DH-38	0.0	0.0	0.0	0.0
AAFC695DH-39	0.0	0.0	0.0	0.0
AAFC695DH-40	0.0	0.0	0.0	0.0

	DSI			
Lines/ Pathotypes	3A	3D	3H	5X
AAFC695DH-41	100.0	83.3	100.0	100.0
AAFC695DH-42	100.0	100.0	83.3	100.0
AAFC695DH-43	0.0	0.0	0.0	0.0
AAFC695DH-44	100.0	100.0	100.0	100.0
AAFC695DH-45	75.0	83.3	100.0	100.0
AAFC695DH-46	0.0	0.0	0.0	0.0
AAFC695DH-47	100.0	100.0	100.0	100.0
AAFC695DH-48	0.0	0.0	0.0	0.0
AAFC695DH-49	0.0	0.0	0.0	30.0
AAFC695DH-50	100.0	100.0	100.0	100.0
AAFC695DH-51	100.0	100.0	100.0	100.0
AAFC695DH-52	0.0	0.0	0.0	0.0
AAFC695DH-53	100.0	100.0	100.0	100.0
AAFC695DH-54	0.0	0.0	0.0	0.0
AAFC695DH-55	100.0	100.0	100.0	100.0
AAFC695DH-56	83.3	100.0	100.0	100.0
AAFC695DH-57	0.0	0.0	0.0	0.0
AAFC695DH-58	0.0	0.0	0.0	0.0
AAFC695DH-59	0.0	0.0	0.0	0.0
AAFC695DH-60	0.0	0.0	0.0	0.0
AAFC695DH-61	100.0	100.0	100.0	100.0

	DSI			
Lines/ Pathotypes	3A	3D	3H	5X
AAFC695DH-62	100.0	100.0	100.0	100.0
AAFC695DH-63	100.0	100.0	100.0	100.0
AAFC695DH-64	100.0	100.0	100.0	100.0
AAFC695DH-65	0.0	0.0	0.0	0.0
AAFC695DH-66	0.0	0.0	0.0	0.0
AAFC695DH-67	0.0	0.0	0.0	50.0
AAFC695DH-68	100.0	100.0	100.0	100.0
AAFC695DH-69	100.0	100.0	100.0	100.0
AAFC695DH-70	100.0	100.0	100.0	100.0
AAFC695DH-71	0.0	0.0	0.0	0.0
AAFC695DH-72	0.0	0.0	0.0	0.0
AAFC695DH-73	0.0	0.0	0.0	0.0
AAFC695DH-74	0.0	0.0	0.0	0.0
AAFC695DH-75	100.0	100.0	100.0	100.0
AAFC695DH-76	100.0	100.0	100.0	100.0
AAFC695DH-77	0.0	0.0	0.0	0.0
AAFC695DH-78	100.0	100.0	100.0	100.0
AAFC695DH-79	0.0	0.0	0.0	0.0
AAFC695DH-80	100.0	100.0	100.0	100.0
AAFC695DH-81	100.0	100.0	100.0	100.0
AAFC695DH-82	0.0	0.0	0.0	0.0

	DSI			
Lines/ Pathotypes	3A	3D	3H	5X
AAFC695DH-83	100.0	100.0	100.0	100.0
AAFC695DH-84	100.0	100.0	100.0	100.0
AAFC695DH-85	0.0	0.0	0.0	0.0
AAFC695DH-86	0.0	0.0	0.0	0.0
AAFC695DH-87	100.0	100.0	100.0	100.0
AAFC695DH-88	0.0	0.0	0.0	0.0
AAFC695DH-89	100.0	100.0	100.0	100.0
AAFC695DH-90	0.0	0.0	0.0	0.0
AAFC695DH-91	0.0	0.0	0.0	0.0
AAFC695DH-92	0.0	0.0	0.0	0.0
AAFC695DH-93	100.0	100.0	100.0	100.0
AAFC695DH-94	0.0	0.0	0.0	0.0
AAFC695DH-95	0.0	0.0	0.0	0.0
AAFC695DH-96	0.0	0.0	0.0	20.0
AAFC695DH-97	100.0	100.0	100.0	100.0
AAFC695DH-98	100.0	100.0	100.0	100.0
AAFC695DH-99	0.0	0.0	0.0	0.0
AAFC695DH-100	0.0	0.0	0.0	0.0
AAFC695DH-101	0.0	0.0	0.0	0.0
AAFC695DH-102	100.0	100.0	100.0	100.0

Table S5. 1**.** Sequence information for the parental *Brassica napus* lines DH16516, cultivar 'Mendel' and a 'Mendel' doubled haploid (DH) population derived from the F_1 of DH16516 × Mendel.

Parents/ Population	Total number of sequences	Assembled reads	Assembly (%)	Total SNPs	SNPs/ 1000 assembled reads	%Template coverage	Fold coverage (Whole Genome)
DH16516	219.9×10^6	188.4×10^6	92.1	1.20 M	63.3	91.06	0.911
Mendel	9.89×10^6	8.79×10^6	88.9	0.40 M	45.1	5.57	0.056
Mendel DH	704.5×10^6	635.6×10^6	90.2	37.9 M	59.7	4.21	0.042

Table S5. 2**.** Information on 2642 SNP markers used in QTL IciMapping to identify and map a putative clubroot resistance gene in Mendel double haploid population derived from the F_1 of DH16516 × Mendel.

Chromosomes	Number of SNPs	Length (cM)	Missing (%)	% of 'a'	% of 'b'	a:b (Approx.)	Interval (ave.) (cM)	# of distorted markers
ZS_A01	26	200	9.32	43.3	47.4	1:1	7.69	25
ZS_A02	192	236	2.92	68.8	28.3	2:1	1.22	169
ZS_A03	317	655.1	3.98	66.6	29.5	2:1	2.07	280
ZS_A04	72	174	1.91	48	50.1	1:1	2.41	23
ZS_A05	154	145	3.23	66.2	30.6	2:1	0.94	135
ZS_A06	127	226	2.57	66.7	30.8	2:1	1.78	109
ZS_A07	134	262.8	4.1	64.9	31	2:1	1.96	89
ZS_A08	129	204.5	3.93	32.3	63.8	1:2	1.59	119
ZS_A09	97	466.8	4.01	36.9	59.1	1:2	4.81	84
ZS_A10	252	362	3.8	74.5	21.7	3:1	1.44	252
ZS_C01	169	298.8	7.46	51.5	41	1:1	1.77	45
ZS_C02	338	388.6	3.5	65.3	31.2	2:1	1.15	336
ZS_C03	111	343.8	4.42	38.4	57.2	1:1	3.09	49

Chromosomes	Number of SNPs	Length (cM)	Missing (%)	% of 'a'	% of 'b'	a:b (Approx.)	Interval (ave.) (cM)	# of distorted markers
ZS_C04	117	406	2.76	55.2	42.1	1:1	3.47	79
ZS_C05	100	335.2	4.38	30.7	64.9	1:2	3.35	93
ZS_C06	49	156.8	6.67	27.1	66.2	1:2	3.2	49
ZS_C07	12	124.9	7.79	9.06	83.2	1:9	10.4	12
ZS_C08	100	427.5	4.49	50.7	44.8	1:1	4.26	81
ZS_C09	146	420.6	4.26	51.9	43.9	1:1	2.88	90

Table S5. 3. Disease Severity Index (DSI) of 137 ‘Mendel’ doubled haploid (DH) lines, derived from the F_1 of DH16516 × Mendel, following inoculation with *Plasmodiophora brassicae* pathotypes 3D, 5C, and 8J.

	DSI		
DH Lines/Pathotypes	3D	5C	8J
MendelDH-01	0.0	0.0	0.0
MendelDH-02	0.0	0.0	0.0
MendelDH-03	100.0	100.0	100.0
MendelDH-04	0.0	0.0	0.0
MendelDH-07	0.0	0.0	0.0
MendelDH-10	0.0	0.0	0.0
MendelDH-11	0.0	0.0	0.0
MendelDH-12	0.0	0.0	12.5
MendelDH-13	0.0	0.0	0.0
MendelDH-15	0.0	0.0	0.0
MendelDH-16	0.0	0.0	0.0
MendelDH-17	100.0	100.0	100.0
MendelDH-18	0.0	0.0	0.0
MendelDH-20	0.0	16.7	10.0
MendelDH-21	100.0	100.0	100.0
MendelDH-22	100.0	100.0	100.0
MendelDH-23	0.0	0.0	0.0
MendelDH-24	100.0	100.0	100.0
MendelDH-25	100.0	100.0	100.0

	DSI		
DH Lines/Pathotypes	3D	5C	8J
MendelDH-27	0.0	0.0	0.0
MendelDH-28	100.0	100.0	100.0
MendelDH-29	0.0	0.0	0.0
MendelDH-30	0.0	0.0	0.0
MendelDH-31	100.0	100.0	100.0
MendelDH-32	0.0	0.0	0.0
MendelDH-34	100.0	100.0	100.0
MendelDH-35	0.0	0.0	0.0
MendelDH-36	0.0	50.0	16.7
MendelDH-37	0.0	0.0	0.0
MendelDH-38	0.0	0.0	0.0
MendelDH-39	100.0	100.0	100.0
MendelDH-40	100.0	100.0	100.0
MendelDH-41	100.0	100.0	100.0
MendelDH-42	0.0	0.0	0.0
MendelDH-43	0.0	0.0	0.0
MendelDH-44	11.1	40.0	0.0
MendelDH-45	0.0	0.0	0.0
MendelDH-46	100.0	100.0	100.0
MendelDH-47	100.0	100.0	100.0
MendelDH-48	0.0	0.0	16.7

	DSI		
DH Lines/Pathotypes	3D	5C	8J
MendelDH-49	0.0	0.0	0.0
MendelDH-53	0.0	0.0	0.0
MendelDH-54	0.0	0.0	0.0
MendelDH-55	0.0	0.0	0.0
MendelDH-56	0.0	0.0	0.0
MendelDH-57	0.0	16.7	14.3
MendelDH-58	0.0	0.0	0.0
MendelDH-59	0.0	0.0	0.0
MendelDH-60	0.0	0.0	12.5
MendelDH-61	0.0	0.0	0.0
MendelDH-62	0.0	0.0	0.0
MendelDH-63	0.0	0.0	0.0
MendelDH-64	0.0	0.0	0.0
MendelDH-65	0.0	0.0	0.0
MendelDH-67	0.0	0.0	0.0
MendelDH-68	0.0	0.0	0.0
MendelDH-69	50.0	0.0	0.0
MendelDH-73	0.0	0.0	0.0
MendelDH-78	0.0	0.0	0.0
MendelDH-80	0.0	0.0	0.0
MendelDH-81	0.0	0.0	0.0

	DSI		
DH Lines/Pathotypes	3D	5C	8J
MendelDH-82	0.0	0.0	0.0
MendelDH-84	0.0	0.0	0.0
MendelDH-85	50.0	0.0	0.0
MendelDH-86	0.0	0.0	0.0
MendelDH-88	0.0	0.0	0.0
MendelDH-89	0.0	0.0	0.0
MendelDH-90	0.0	0.0	0.0
MendelDH-92	0.0	0.0	0.0
MendelDH-94	0.0	0.0	0.0
MendelDH-96	0.0	0.0	0.0
MendelDH-97	0.0	0.0	0.0
MendelDH-98	0.0	0.0	0.0
MendelDH-99	0.0	0.0	0.0
MendelDH-100	18.2	0.0	0.0
MendelDH-101	0.0	33.3	0.0
MendelDH-102	0.0	0.0	0.0
MendelDH-103	8.3	0.0	0.0
MendelDH-104	0.0	0.0	0.0
MendelDH-105	0.0	0.0	0.0
MendelDH-106	0.0	0.0	0.0
MendelDH-107	0.0	0.0	0.0

	DSI		
DH Lines/Pathotypes	3D	5C	8J
MendelDH-108	0.0	0.0	0.0
MendelDH-109	100.0	100.0	100.0
MendelDH-110	0.0	0.0	0.0
MendelDH-111	0.0	0.0	0.0
MendelDH-112	0.0	0.0	0.0
MendelDH-113	16.7	0.0	0.0
MendelDH-114	0.0	0.0	0.0
MendelDH-115	100.0	100.0	100.0
MendelDH-116	33.3	0.0	16.7
MendelDH-117	0.0	0.0	0.0
MendelDH-118	100.0	100.0	100.0
MendelDH-119	0.0	0.0	0.0
MendelDH-120	50.0	0.0	0.0
MendelDH-121	0.0	0.0	0.0
MendelDH-122	0.0	0.0	0.0
MendelDH-123	100.0	100.0	100.0
MendelDH-124	100.0	100.0	100.0
MendelDH-125	0.0	0.0	0.0
MendelDH-126	0.0	0.0	0.0
MendelDH-127	100.0	100.0	100.0
MendelDH-128	0.0	0.0	0.0

	DSI		
DH Lines/Pathotypes	3D	5C	8J
MendelDH-129	0.0	0.0	0.0
MendelDH-130	100.0	100.0	100.0
MendelDH-131	0.0	0.0	0.0
MendelDH-132	0.0	0.0	0.0
MendelDH-133	100.0	100.0	100.0
MendelDH-134	0.0	0.0	0.0
MendelDH-135	0.0	0.0	0.0
MendelDH-136	0.0	0.0	44.4
MendelDH-137	0.0	0.0	16.7
MendelDH-138	0.0	0.0	0.0
MendelDH-139	0.0	0.0	0.0
MendelDH-140	0.0	0.0	0.0
MendelDH-141	0.0	0.0	0.0
MendelDH-142	0.0	0.0	0.0
MendelDH-144	0.0	0.0	25.0
MendelDH-145	0.0	0.0	0.0
MendelDH-146	0.0	0.0	0.0
MendelDH-147	0.0	0.0	0.0
MendelDH-148	0.0	0.0	0.0
MendelDH-149	0.0	0.0	0.0
MendelDH-150	100.0	100.0	100.0

	DSI		
DH Lines/Pathotypes	3D	5C	8J
MendelDH-151	0.0	0.0	0.0
MendelDH-153	0.0	0.0	0.0
MendelDH-154	0.0	0.0	0.0
MendelDH-155	100.0	100.0	100.0
MendelDH-156	100.0	100.0	100.0
MendelDH-157	0.0	0.0	0.0
MendelDH-158	0.0	0.0	0.0
MendelDH-159	100.0	100.0	100.0
MendelDH-160	0.0	0.0	0.0
MendelDH-161	0.0	0.0	0.0
MendelDH-162	0.0	0.0	16.7
MendelDH-163	0.0	0.0	0.0
MendelDH-164	0.0	0.0	0.0

Table S5. 4. Disease Severity Index (DSI) of the sub-population with 60 ‘Mendel’ doubled haploid (DH) lines, derived from the F_1 of DH16516 × Mendel, following inoculation with *Plasmodiophora brassicae* pathotype 3H.

DH Lines	DSI
MendelDH-03	20.0
MendelDH-04	0.0
MendelDH-07	0.0
MendelDH-10	0.0
MendelDH-11	0.0
MendelDH-12	0.0
MendelDH-13	0.0
MendelDH-15	0.0
MendelDH-16	0.0
MendelDH-18	0.0
MendelDH-20	0.0
MendelDH-23	0.0
MendelDH-27	0.0
MendelDH-29	0.0
MendelDH-30	0.0
MendelDH-32	0.0
MendelDH-35	42.9
MendelDH-37	41.7
MendelDH-38	46.7
MendelDH-42	50.0

DH Lines	DSI
MendelDH-48	50.0
MendelDH-53	42.9
MendelDH-54	0.0
MendelDH-56	0.0
MendelDH-61	37.5
MendelDH-62	48.1
MendelDH-65	44.4
MendelDH-73	37.5
MendelDH-85	0.0
MendelDH-86	0.0
MendelDH-89	0.0
MendelDH-92	0.0
MendelDH-94	0.0
MendelDH-96	0.0
MendelDH-99	16.7
MendelDH-100	20
MendelDH-101	55.6
MendelDH-103	38.9
MendelDH-104	33.3
MendelDH-105	57.1
MendelDH-106	0.0
MendelDH-107	0.0

DH Lines	DSI
MendelDH-108	0.0
MendelDH-110	0.0
MendelDH-111	0.0
MendelDH-114	28.6
MendelDH-119	0.0
MendelDH-120	0.0
MendelDH-121	0.0
MendelDH-122	0.0
MendelDH-126	0.0
MendelDH-128	0.0
MendelDH-129	0.0
MendelDH-131	100.0
MendelDH-133	100.0
MendelDH-150	100.0
MendelDH-155	100.0
MendelDH-156	100.0
MendelDH-159	100.0
MendelDH-163	51.9

Supplemental Figures

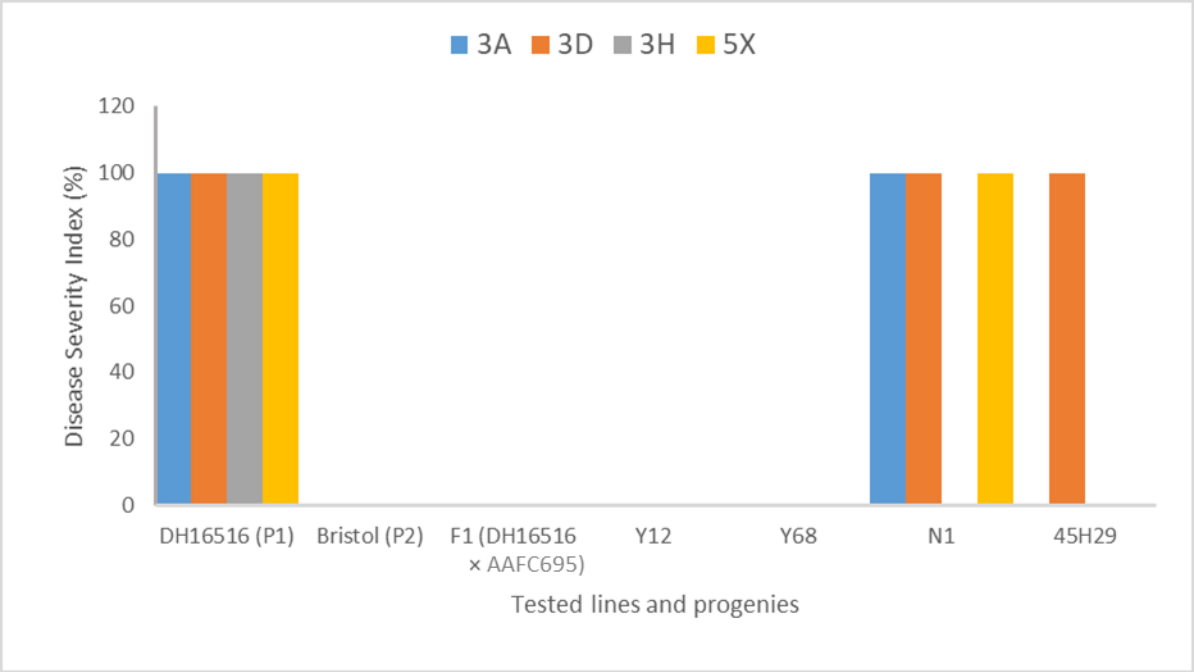

Figure S4. 1. Reaction of the *Brassica napus* parental lines DH16516 and 'AAFC695', their F_1 progeny, the resistant checks Y12 and Y68, and the susceptible checks DH16516, NRC11-24 and '45H29', to inoculation with *Plasmodiophora brassicae* pathotypes 3A, 3D, 3H and 5X.

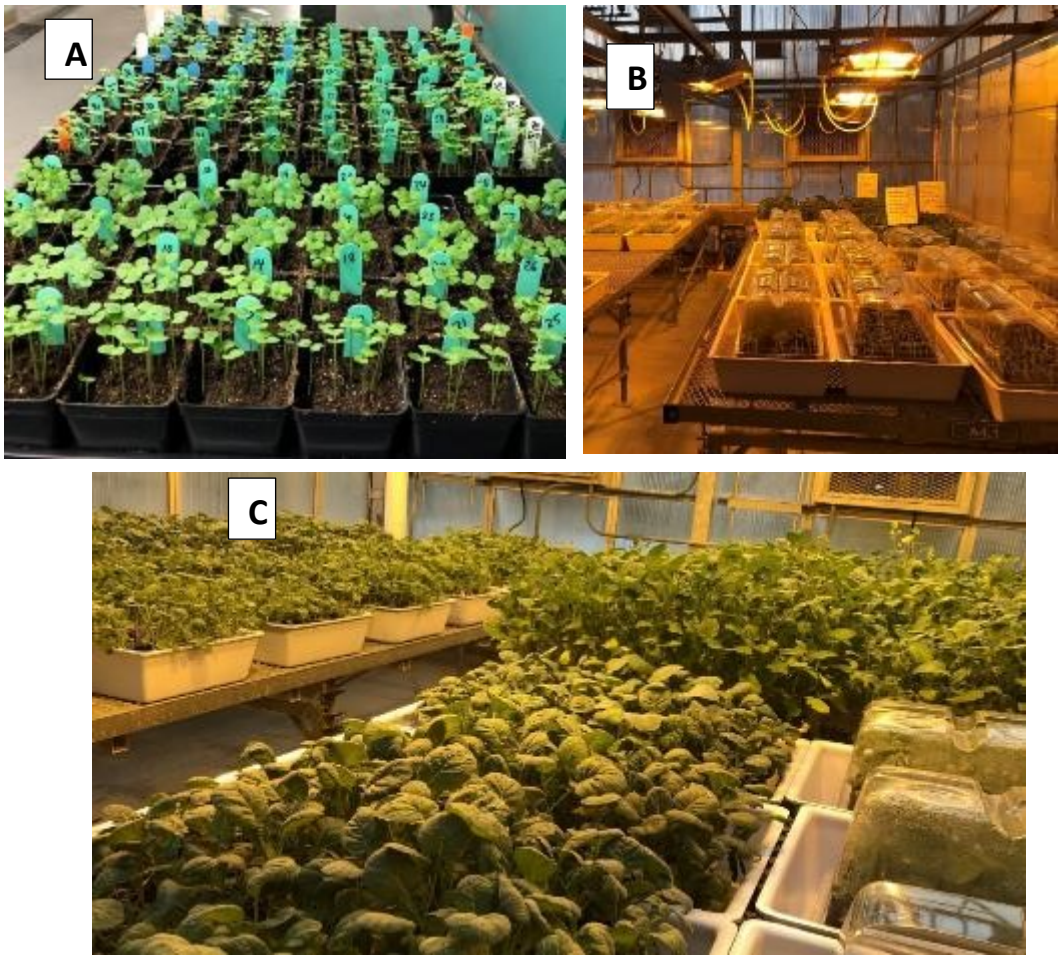

Figure S4. 2**.** Phenotyping of *Plasmodiophora brassicae*-inoculated seedlings of *Brassica napus* AAFC695DH population, derived from DH16516 × AAFC695), under controlled conditions at the Agriculture and Agri-Food Canada Saskatoon Research and Development Centre, Saskatoon, SK. (A): Seedlings prior to inoculation; (B): Seedlings in the greenhouse after inoculation (covered with plastic lids to preserve moisture); and (C): Growing seedlings prior to clubroot disease scoring.

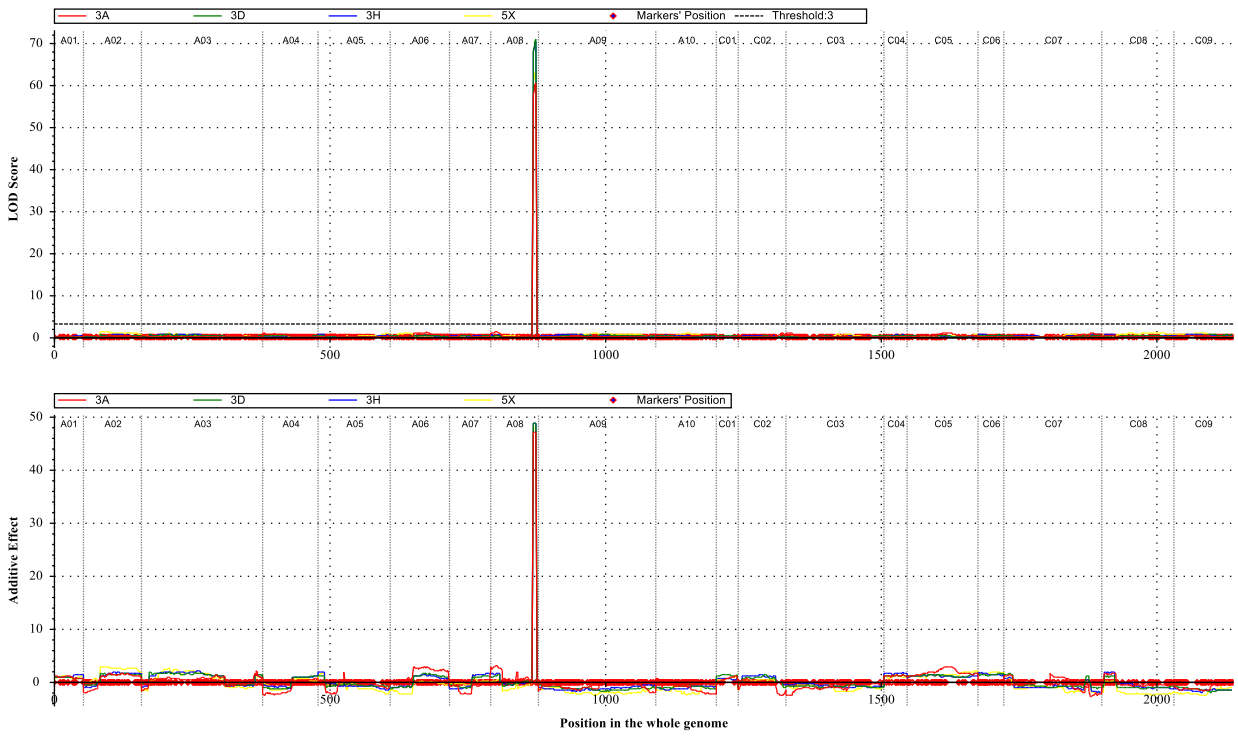

Figure S4. 3**.** The identified QTL on chromosome A08 and additive effect of the QTL considering the whole genome, 19 chromosomes, of *Brassica napus* AAFC695DH population derived from DH16516 × AAFC695.

Figure S5. 1. Disease scoring scale, based on Kuginuki et al. (1999), where: 0 = no galls, 1 = a few small galls, 2 = moderate galls, and 3 = severe galling on the roots.

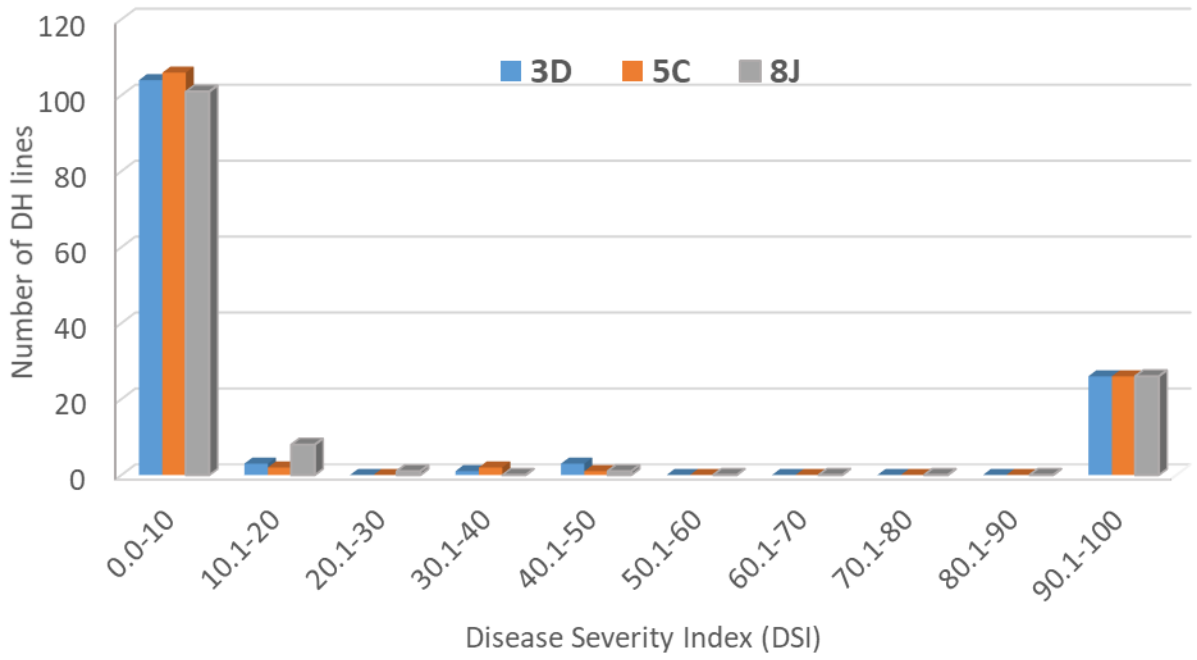

Figure S5. 2**.** Clubroot disease severity index of 137 lines of a *Brassica napus* 'Mendel' doubled haploid population, derived from DH16516 × Mendel) after inoculation with *Plasmodiophora brassicae* pathotypes 3D, 3H, 5C, and 8J.

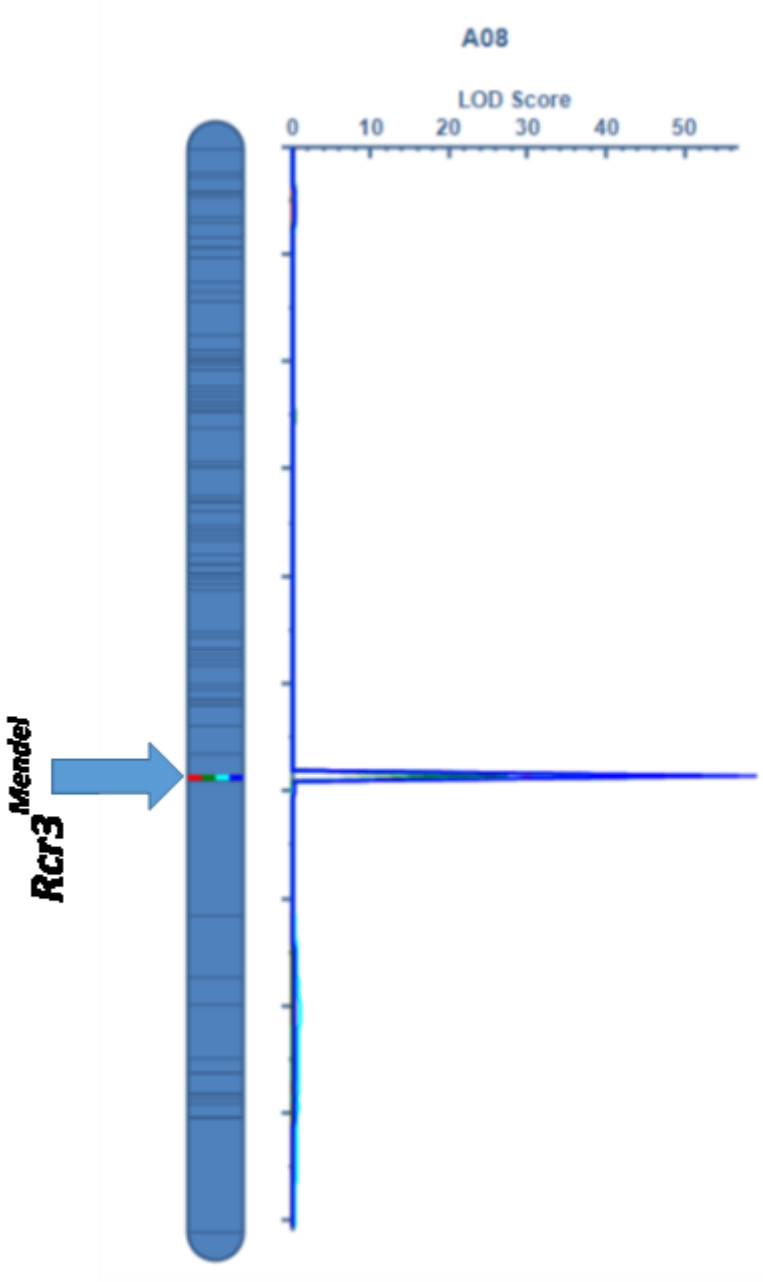

Figure S5. 3**.** The *Rcr3*^{Mendel} region on chromosome A08 with corresponding LOD values.

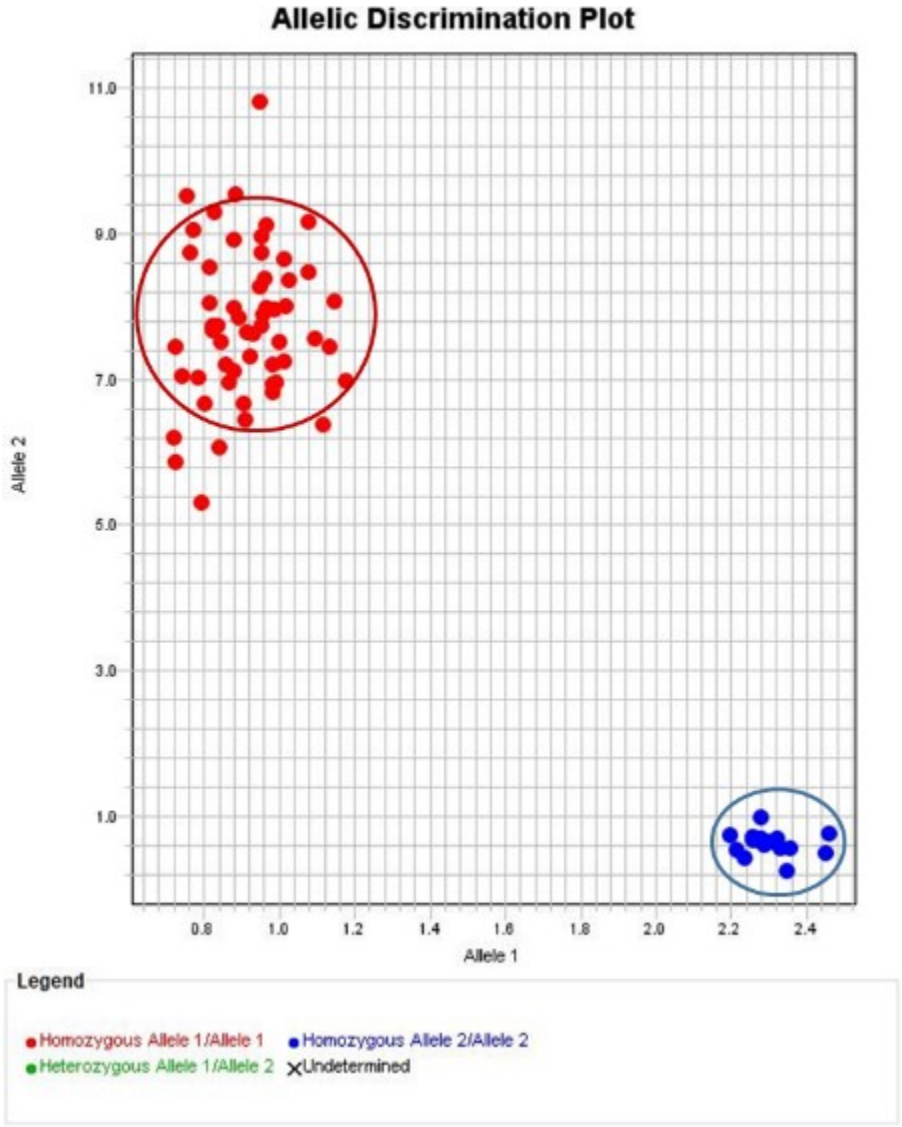

Figure S5. 4**.** Allelic discrimination plot from KASP SNP markers analysis of 137 doubled haploid lines developed from the F_1 of a *Brassica napus* DH16516 × 'Mendel' cross. The red cluster indicates the homozygous alleles from resistant lines and the blue cluster indicates the homozygous alleles from susceptible lines.

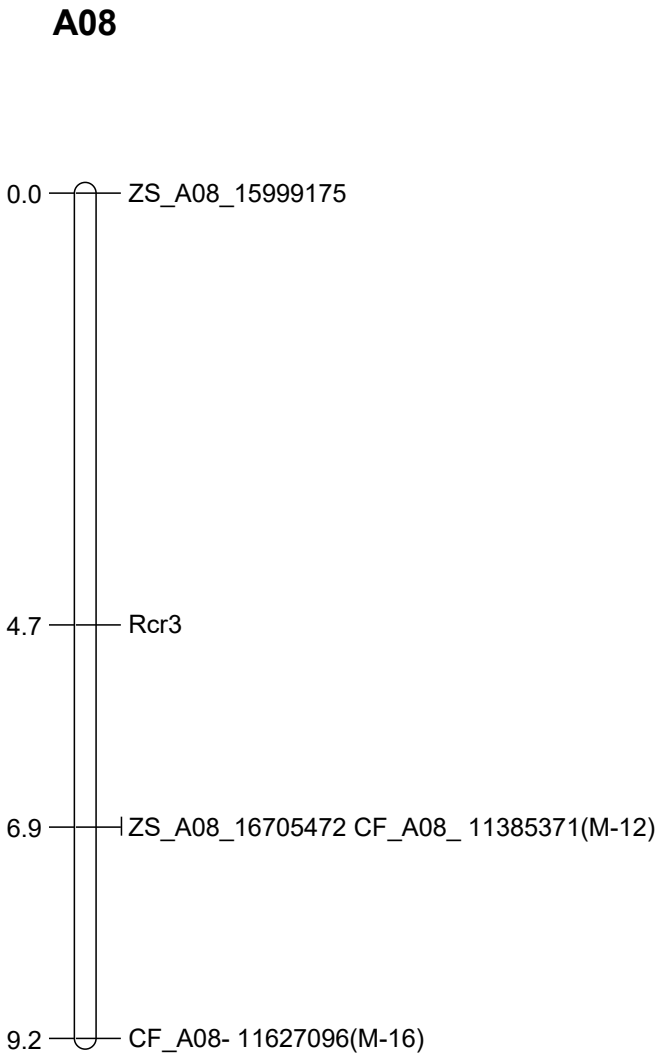

Figure S5. 5. KASP analysis confirms the locus *Rcr3* is the same locus as of $Rcr3^{Mendel}$. In the figure, the M12 and M16 is the *Rcr3* Marker identified from *B. rapa* v1.5 and converted to v.3.0 (Chiifu), whereas ZS_A08_15999175 and ZS_A08_16705472 are the $Rcr3^{Mendel}$ markers identified through *B. napus* reference gene ZS11.