Studies of pathogenicity in *Plasmodiophora brassicae* and segregation of clubroot resistance genes from *Brassica rapa* subsp. *rapifera*

by

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Abstract

The planting of clubroot resistant (CR) canola (*Brassica napus*) is the most effective method to manage clubroot, a soilborne disease caused by *Plasmodiophora brassicae*. In recent years, many *P. brassicae* isolates capable of overcoming resistance have been detected, often in mixtures with avirulent isolates. To improve understanding of the effect of low concentrations of virulent isolates on host resistance, three CR canola cultivars ('45H29', 'L135C' and 'L241C') were inoculated with pairs of isolates representing virulent/avirulent pathotypes (2*/2, 3*/3 and 5*/5) of *P. brassicae*, collected after or before the introduction of CR canola, respectively. Clubroot severity was significantly higher in all nine experimental treatments (low virulent + high avirulent) than in the negative control NC1 (high avirulent), and higher in seven of nine experimental treatments than in the negative control NC2 (low virulent). Disease severity was positively correlated with *P. brassicae* biomass *in planta*, as determined by quantitative PCR analysis 28 - 35 days after inoculation (dai). These results suggest that low concentrations of virulent isolates compromised the clubroot resistance in canola, facilitating infection by avirulent isolates.

In a second study, the expression of 205 *P. brassicae* genes encoding putative secreted proteins was compared following inoculation of the canola '45H29' with pathotypes 5I (avirulent) and 5X (virulent) of the pathogen. Sixteen of these genes were differentially expressed at 14 dai, and additional monitoring at multiple timepoints (7, 14 and 21 dai) indicated that, collectively, 12 of the 16 genes were upregulated in pathotype 5X. The relative expression of the same 16 genes was also compared in the interaction between the canola 'Westar' and pathotype 5I (virulent on 'Westar'), with 12 of the genes showing similar expression patterns as in the '45H29'/5X interaction. Given their common expression patterns in two compatible

interactions, it is possible that these genes play a role in clubroot pathogenesis.

In a third and final study, clubroot resistance was introgressed from the European Clubroot Differential (ECD) 02 into the *B. rapa* accessions CR 2599 and CR 1505 ('Emma'), and into the *B. napus* accession CR 1054 ('Westar'). The distorted segregation ratios suggest that the two resistance genes are on different chromosomes and that two genes interact in an epistatic manner to confer resistance. Genotyping was conducted with 144 PCR-based markers in two of the three F_2 populations. Linkage and QTL analysis with the polymorphic markers identified two QTLs on chromosome A03 associated with resistance to *P. brassicae* pathotypes 5X and 5G in Popl#1, whereas only the second QTL on chromosome A03 was associated with resistance to these pathotypes in Popl#2. The two QTLs clustered in genomic regions on the A03 chromosome of *B. rapa* where the *CRa*/*CRb*^{Kato} gene(s) are mapped. In addition, the *Crr1* gene on the A08 chromosome of *B. rapa* was detected in the two populations. Therefore, the phenotypic and molecular data confirm the existence two CR genes in ECD 02.

Preface

This thesis is an original work by Mr. Junye Jiang, who conducted the experiments and wrote the first drafts of all the chapters. Mr. Jiang's supervisors, Dr. Stephen E.Strelkov and Dr. Sheau-Fang Hwang, reviewed and edited each chapter. The chapters were also reviewed by Dr. Rudolph Fredua-Agyeman, a member of Mr. Jiang's supervisory committee. Dr. Fredua-Agyeman also assisted with the data analysis and provided feedback and constructive suggestions on the experimental design of some of the studies. Dr. Robert Conner (Agriculture and Agri-Food Canada) and Mr. George Turnbull (Alberta Agriculture and Forestry) also reviewed Chapter 3 and provided suggestions for improvements. The Brassica accessions used in the experiments described in this thesis were obtained from the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany. The field and single-spore isolates of the clubroot pathotgen, *Plasmodiophora brassicae*, were from the research collections of Drs. Strelkov and Hwang. Technical staff and students from the Plant Pathology Lab, University of Alberta, assisted with the inoculation experiments described in Chapters 2 and 4. Financial support for this research came through grants awarded by Agriculture and Agri-Food Canada and the Canola Council of Canada through the Growing Forward 2 and Canadian Agricultural Partnership (CAP) programs to Drs. Strelkov, Hwang, and Fredua-Agyeman. Additional funding for earlier parts of this work was provided by the Alberta Crop Industry Development Fund. In-kind support, including access to facilities and equipment, was provided by the University of Alberta and Alberta Agriculture and Forestry.

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Symbols and Abbreviations

χ^2 Chi-square
µL Microliter
AFLP Amplified fragment length polymorphism
ANOVA Analyses of variance
BC Backcross
cDNA Complementary DNA
CIM Composite internal mapping
cM Centimorgan
CR Clubroot resistance
Ct Cycle threshold
CTAB Cetyltrimethyl ammonium bromide
DAI Days after inoculation
DH Doubled haploid
DI Disease incidence
DSI Disease severity index
ECD European clubroot differentials
ET Experimental treatment
GLM General linear model
HA Hectare
HT Herbicide tolerant
ICIM-ADD Inclusive composite interval mapping-additive
Indel Insertions and deletions
LOD Logarithm of odds
MAS Marker-assisted selection
MIM Multiple interval mapping
mM Millimoles per liter
MT Metric tonne
NAD(P)H Nicotinamide adenine dinucleotide (phosphate)-reduced
NC Negtive control
nM Nanomoles per liter
Θ Recombination fraction
PAMP Pathogen-associated molecular pattern
PC Positive control
PCR Polymerase chain reaction
qPCR Quantitative PCR
QTL Quantitative trait loci
RAPD Random amplified polymorphic DNA
RFLP Restriction fragment length polymorphism

RIL Recombinant inbred lines
RQ Relative quantity
SIM Single interval mapping
SMA Single marker analysis
SNP Single nucleotide polymorphism
SSCP Single-stranded conformation polymorphism
SSR Simple sequence repeat

Chapter 1: Introdcution and Literature Review

1.1 Introduction to the Brassicas

The Brassicas include the diploid species *B. nigra* L. (2n = 16, BB), *B. oleracea* L. (2n = 18, CC) and *B. rapa* L. (2n = 20, AA), and the amphidiploids *B. carinata* (A.) Braun (2n = 34, BBCC), *B. juncea* L. (2n = 36, AABB) and *Brassica napus* L. (2n = 38, AACC). The genomic relationships between these six species are represented by the triangle of U (1935), which illustrates how the amphidiploids were derived by hybridization between pairs of diploid species (Figure 1.1). Among the most important end-uses of the Brassicas is as oilseed crops. The oilseed Brassicas have a high seed oil content (35% to 45%), and rapeseed (*B. napus*) is the third most important edible oil source worldwide, preceded only by soybean and palm (Hua et al. 2016). Historically, however, Brassica oil usually contained high levels of erucic acids and glucosinolates, making it undesirable for human consumption.

1.2 Development of Canola

By applying conventional breeding techniques, researchers from Agriculture and Agri-Food Canada and the University of Manitoba developed "double-zero rapeseed" (referring to low erucic acid and low glucosinolate) in the 1970s. The term 'canola' ('can' for Canada and 'ola' for oil) refers to oilseed Brassicas (*B. napus*, *B. rapa* and *B. juncea*) with an erucic acid content in the fatty acid profile of < 2% and a glucosinolate content in the meal of < 30 μmol/g (Ali et al. 2009). The 'meal' refers to the leftover material following oil extraction from the seeds, which is used as an animal feed. The average oil content is 45% (Velasco et al. 1999; Ghazani and Marangoni 2013). Canola oil has a good balance of fatty acids: 50 - 66 % oleic, 6 - 14 % alpha linoleic (Ghazani and Marangoni 2013) and is a rich source of Omega 3 and Omega 6 linolenic acids. It has no *Trans*-fat or cholesterol, is a good source of vitamin E, and has monounsaturated

fatty acid levels comparable with safflower and sunflower oils (Canola council of Canada, 2019). These characteristics make canola oil a healthy choice for human consumption and it also can be used in the chemical and pharmaceutical industries (Friedt and Snowdon 2009). Following extraction of the oil, the leftover seed material ('meal') serves a high quality protein source for livestock (Canola Council of Canada 2019). Most canola cultivars in Canada at present are of the *B. napus* type.

Some canola genotypes require vernalization to induce flowering while others do not. Spring-type canola cultivars do not need vernalization and are grown mainly in western Canada (Zanewich and Rood 1995). Winter-type canola is grown in Europe, where the winter (November to March) is not as harsh as in western Canada. The winter-types have a longer vegetative period and better yields. In several East Asian countries, including China and Japan, canola is usually grown in the winter where the mild conditions support moderate vernalization, which may not even be necessary for flowering. These constitute the semi-winter canola types (Mendham and Robertson 2016).

1.3 Economic Importance of Canola

In 2016, canola production was 18.4M MT and contributed over CAD $26.7 billion to the Canadian economy. The canola industry also provides employment for over 250,000 people including farmers, seed processors, transporters and researchers (Canola Council of Canada, 2019). The Canola Council of Canada has set an ambitious production target of 26M MT by 2025. To achieve this target, yield must increase from the present 2844.7 kg/ha to 3497.1 kg/ha, since canola acreage is expected to increase only marginally (8.26 to 8.90 million ha) as a result of competing demands on the land. The Canola Council of Canada (2019) has identified five key

areas of focus to realize the 2025 production target: genetic improvement, plant establishment, fertility management, integrated pest management and harvest management.

1.4 Canola Agronomy and Pests

Continued improvements in the productivity of canola will require a better understanding of plant development, establishment of uniform populations, optimal fertilizer application, early control of weeds, insects and diseases, careful scouting, and study of the conditions that lead to higher losses. In general, canola yield and quality are highest under favorable environmental conditions.

1.4.1. Environmental factors affecting canola growth

Environmental factors affecting canola growth include temperature, photoperiod, water, and soil conditions (texture, pH, drainage, organic matter, and inorganic salts).

Temperature and photoperiod

Temperature plays a very important role in the growth and development of canola. Seed germination, seedling emergence, the rate of root, leaf, stem, pod and seed development, progression to flowering, yield, the incidence and severity of diseases all are influenced by temperature (Mendham and Robertson 2016). Canola performs best in temperate regions with temperatures around 20^{o}C; winter-type canola also requires a cold treatment or vernalization period of 5^{o}C for 25 days to induce flowering (Robertson et al. 2002). Canola has the ability to tolerate significant fluctuations in temperature, but very high temperature stress (38^{o}C) reduces seed weight and hence yield, while also decreasing the oil/protein ratio (Aksouh-Harradj et al. 2006). Relative to optimal temperatures (22/18^{o}C), higher temperatures (28/24^{o}C) lead to reduced growth and development of the seedlings (Qaderi 2011). This is because higher

temperatures can reduce photosynthesis while increasing transpiration and evaporation; consequently, crop biomass is reduced (Nobel 2009; Qaderi and Reid 2009). From a global standpoint, climate change and the associated increases in average temperatures will reduce the yield and quality of canola in warmer regions, but may be beneficial for production in cooler areas (Pritchard and Amthor 2005).

In addition to temperature, photoperiod affects the length of time required from plant emergence to floral initiation. In canola, an increase in the photoperiod, due to longer days, reduces the number of days required to reach maturity (Mendham and Robertson 2016). The optimal photoperiod is between 10.8-16.3 h per day after seeding emergence (Robertson et al 2002). Given the impacts of temperature and photoperiod, sowing of the crop at an optimal date is very important for optimal canola growth and development. Planting later, when temperatures are warmer, may favor more rapid growth and emergence of the seedlings (Ozer 2003), but sowing too late may result in insufficient time for full crop development/harvest before the onset of winter. The optimal date to sow canola depends on specific growing conditions, seed quality and the cultivar being planted. Indeed, a recent Canadian study indicated that early sowing is associated with higher yields and seed oil concentration (Ma et al. 2016).

Water supply

Water is important for photosynthesis, respiration and transpiration, and water availability is very important for canola production (Wright et al. 1996). Available water includes stored soil water before sowing, rainfall during crop growth and water applied manually. Water efficiency depends on evaporative rate and soil properties. It also depends on whether water is applied at optimal or critical times, such as just after seeding or flowering. Water management strategies should therefore optimize water supply at critical periods in the development of the plant. It was

reported by the Canola Council of Canada (2019) that on the Canadian Prairies, canola grows best on thick black and grey soils rich in humus. A hardpan layer of clay often restricts root development and water infiltration and can lead to reduced moisture in the topsoil. This affects the upward movement of water and nutrients resulting in stunted growth. Generally, there is an increase in seed yield and oil content with sufficient water supply (Al-Jaloud et al. 1996). In many parts of the world, irrigation has been used to compensate for insufficient rainfall (McCaffery 2004; Bauder 2006). In much of the canola growing regions of Canada, however, the crop is grown under dryland conditions. The impact of water stress on canola can vary. For instance, Tesfamariam (2010) found that canola at the flowering stage was more sensitive to water stress than canola at the vegetative or seed-filling stages. An increased application of water and fertilizer also improved the efficiency of biomass accumulation per unit canola N and canola N per unit of available N supply (Maaz et al 2016).

Soil nutrients

When grown in soils with poor fertility (e.g., solonetzic soils), seed yield and oil content are low (Toogood et al. 1973). Organic matter holds soil (clay, silt and sand) particles together and provides a rich source of nutrients to plants. However, additional nutrient requirements can be met by fertilizer application. Among the essential nutrients (C, H, O, N, P, K, Ca, Mg, S, B, Cl, Cu, Fe, Mn, Mo, Zn), nitrogen (N) is very critical for canola seed yield (Ozer 2003).

Canola needs about 50-70 kg of available nitrogen per tonne of seed yield (Mendham and Robertson 2016). Most nitrogen is taken up by the roots prior to flowering and is translocated to the stems, leaves, pods and seeds. The amount of nitrogen needed for optimal growth is influenced by nitrogen use efficiency (NUE), which is low in *B. napus* (Sylvester-Bradley and Kindred 2009). The application of excessive nitrogen will increase the growth of leaves and

stems, but decrease protein and oil content in the seeds (Tilman 2002; Billen 2013), and potentially result in increases in weed biomass (Blackshaw et al. 2003; Naderi and Ghadiri 2010). Improving NUE is important for enhanced seed yield and quality as well as environmental sustainability. Lynch (2014) stated that root architecture is a lever to optimize the balance between nitrogen absorption and metabolic needs, suggesting that breeding activities should focus on improving root architecture to increase NUE.

Phosphorus, potassium and sulfur are other important macronutrients. Canola requires about 12 kg phosphorus and 20 kg potassium per tonne of seed yield, while sulfur requirement is about 10 kg (Mendham and Robertson 2016). Deficiencies in these macronutrients can cause stunted growth, pale and yellow leaves and very poor pod development. In addition, numerous micronutrients including molybdenum (Mo), manganese (Mg), boron (B) and zinc (Zn) are important for the growth and development of canola. However, manganese toxicity may be an issue in acid soils (Mendham and Robertson 2016).

1.4.2 Weeds and canola

Weed competition is an important threat to canola production (Harker 2001). The crop can be very sensitive to weed competition, especially between seedling emergence and the 4-6 leaves stage (Martin et al 2001). In western Canada, > 95% of the canola acreage is planted to herbicide tolerant (HT) varieties (Canola Council of Canada 2010). HT canola facilitates weed control and can improve canola yields while reducing use of herbicides (Morrison et al. 2016). Nevertheless, weeds are still a major issue in canola, particularly given the rapid spread of herbicide-resistant weed species. Weeds resistant to 22 of 25 known modes of action and 160 formulations of herbicides have been documented (Heap 2016). As such, integrated weed management approaches are required.

Cultivar selection can have a significant impact on weed control. Hybrid canola cultivars are more competitive with weeds than older, open-pollinated cultivars; the hybrid cultivars show better seedling density and improved yields (Harker et al. 2003, 2011). Indeed, hybrid canola was twice as competitive as open-pollinated varieties when wild oat populations were high (Zand and Beckie 2002). Seeding rates can also be manipulated to help manage weeds. Many studies have found that an increased seeding rate can reduce weed biomass, seed weight and seed fecundity (Harker et al. 2003; O'Donovan et al. 2004; Bakhtiari and Saeedipoor 2014). Row spacing is another factor affecting weed competition. Most studies indicate that narrow spacing between rows can reduce weed biomass and increase canola yield (Andersson and Bengtsson 1992; Felton et al. 2004; Borger et al. 2010; Scott et al. 2013). A denser crop stand helps deprive the weeds of light, space and other resources.

1.4.3 Insects and canola

Insect pests represent another threat to canola production. Some of the main insects affecting canola in western Canada include flea beetles (*Phyllotreta*) (Dosdall et al. 1999), alfalfa looper (*Autographa californica*), aphids (*Aphidoidea*), bertha armyworm (*Mamestra configurata*) and the cabbage seedpod weevil (*Ceutorhynchus assimilis*) (Gavloski et al. 2011). Different insect pests may attack the crop at different growth stages; for instance, flea beetles may threaten seedling establishment, whereas as aphids can damage the flowers (Canola Council of Canada 2019; Gu et al. 2007). The application of pesticides is a useful approach to control insect infestations, but excessive usage can result in the development of insecticide resistance in the pest (McDonald et al. 1999). Furthermore, some insecticides may have adverse environmental or non-target effects. As such, an integrated insect pest management approach, which combines

genetic resistance, biological control and cultural control strategies, may be more effective in the long term.

1.4.4 Canola diseases

In addition to weeds and insects, another major constraint to canola production is the occurrence of disease. While *B. napus* and other Brassicas are hosts to a wide range of pathogens, three are particularly relevant to western Canadian cropping systems. Blackleg is caused by the fungus *Leptosphaeria maculans* (Desm.) Ces. et de Not. and is perhaps the most important canola disease on the Prairies. It can cause foliar lesions, but the most destructive symptom is the formation of cankers at the base of the stem, which can girdle the plant and result in lodging and death. While most canola cultivars are resistant or moderately resistant to *L. maculans*, new races of the fungus have been detected in recent years that can overcome some of this resistance and may hamper blackleg management efforts (Kutcher et al. 2006). Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, is another important canola disease. Most cultivars are susceptible to stem rot, and therefore management relies mainly on the application of fungicides at the flowering stage (Bailey et al. 2000; Budge and Whipps 2001). The third of the most important pathogens, *Plasmodiophora brassicae* Woronin, causes clubroot, which is the focus of this dissertation. Hence, the disease and its causal agent will be discussed in detail in the section below.

1.5 Clubroot of Canola

1.5.1 History of clubroot disease

The earliest records of clubroot date back to the 13th century, when symptoms characteristic of the disease were found on Brassica crops in Europe. In the early 19th century, a Scottish researcher ascribed clubroot to “unsatisfactory soil conditions or unbalanced fertilizer practices”

(Dixon 2009a). It was not until later in the 19th century when a Russian biologist M.S. Woronin (1878) identified the causal agent of clubroot as *P. brassicae*. Clubroot was introduced to Canada late in the 19th or early 20th century, and was likely brought to the country by settlers and their crops (Estey 1994; Howard et al. 2010). It soon became widely established on cruciferous vegetables in British Columbia, Ontario, Quebec and the Maritime Provinces, but was not reported on canola until 1997 (Morasse et al. 1997). At that time, the disease was found on canola in Quebec; the first report of clubroot of canola in western Canada was in 2003, when it was identified in 12 fields near Edmonton, Alberta (Tewari et al. 2005). The clubroot outbreak has spread rapidly over the past 15 years, and by 2018 the disease had been documented in 3,044 fields in Alberta (Strelkov et al. 2019). The disease also appears to be spreading into Saskatchewan and Manitoba (Strelkov and Hwang 2014).

1.5.2 Clubroot symptoms

Clubroot is associated with the development of large galls or 'clubs' on the roots of susceptible plants. These malformations interfere with normal root function and the ability of plants to take up water and nutrients from the soil. Consequently, when symptoms are severe, aboveground growth may be stunted, and the plants may wilt under hot conditions. The plants may also become chlorotic and senesce prematurely. In canola, both yield and quality are significantly reduced (Pageau et al. 2006), and in some cases heavily infected crops have not been harvested (see Strelkov and Hwang 2014 for review). Symptom severity reflects in large part the level of pathogen inoculum (resting spores) in the soil. Inoculum concentrations of 1×10^5 resting spores per ml of soil can cause significant clubroot symptoms on susceptible cultivars, clearly distinguishing between resistant and susceptible hosts (Hwang et al. 2017). Very high levels of *P. brassicae* inoculum ($\geq 1 \times 10^6$ resting spores per ml of soil) have been identified in heavily

infested canola fields in Alberta (Strelkov and Hwang 2014). In addition to the inoculum load, environmental conditions may also influence clubroot severity, as will be discussed in Section 1.5.5 below.

1.5.3 Taxonomy and lifecycle of *P. brassicae*

Plasmodiophora brassicae is classified in the Plasmodiophorida within the Rhizaria, a supergroup of mainly unicellular eukaryotes (Bass et al. 2009; Burki et al. 2010; Neuhauser et al. 2014). It is an obligate parasite of the family Brassicaceae, with a lifecycle consisting of three distinct stages: resting spores in the soil, primary (root hair) infection and secondary (cortical) infection. The resting spores are survival structures that can persist in the soil for up to 18 years (Wallenhamar 1999). Environmental factors such as moisture content, soil temperature and the depth to which *P. brassicae* spores are buried in the soil can influence the longevity of spores (Gossen et al. 2014). Any process or activity that moves soil can also move the resting spores. In western Canada, the main mechanism of pathogen dispersal is via the movement of *P. brassicae*-infested soil on farm machinery (Cao et al. 2009). Secondary mechanisms of spread include movement on windborne dust (Rennie et al. 2015) and possibly as an external contaminant on seeds and tubers of various crops (Rennie et al. 2011). Soil temperatures of 24℃ and a pH of 6.0 - 6.7 represent the optimal conditions for the germination of resting spores (Dixon 2009b).

The resting spores produce primary zoospores upon germination, which require a water film to swim in the soil towards host roots. When the primary zoospores encounter root hairs, the primary infection stage of the *P. brassicae* lifecycle is initiated. The zoospores encyst and penetrate the root hairs, forming zoosporangial clusters that eventually give rise to another zoosporic stage, the secondary zoospores (Hwang et al. 2012a). The secondary zoospores are released back into the soil prior to re-infecting the host (Naiki 1984; Feng et al. 2012). This

suggests that zoospores reproduce in large numbers in a very short time during primary infection. Morphologically, primary zoospores from resting spores and secondary zoospores from primary zoosporangia look identical (Ingram and Tommerup 1972; Feng et al. 2012; Feng et al. 2013). Primary infection can occur in susceptible and resistant hosts, and even in some non-host species (Friberg et al. 2006; Deora et al. 2012). Secondary infection, however, is restricted or does not occur in resistant hosts and non-host species. These findings suggest lower host-specificity in primary vs. secondary infection.

Secondary zoospores cause secondary infection, which is associated with colonization of the root cortex by *P. brassicae*. Colonization of the roots results in hypertrophy and hyperplasia of the host tissues, resulting in abnormal growth and the characteristic clubbing of infected roots. During secondary infection, the pathogen produces intracellular secondary plasmodia. These multinucleate structures are eventually into thousands of resting spores. Once the root disintegrates, vast quantities of the resting spores are released into the soil to complete the pathogen lifecycle (Hwang et al. 2012b). Infection of compatible hosts progressively leads to inoculum build-up for succeeding cropping seasons.

1.5.4 Involvement of hormones in disease development

Secondary infection by *P. brassicae* is accompanied by hypertrophy and hyperplasia of the host root tissues, resulting in the development of the characteristic galls associated with clubroot. This abnormal growth is caused by the activity of the plant hormones auxin and cytokinin (Devos et al. 2005; 2006). These hormones regulate cellular division, and stem and root growth. Auxin is linked to an increase in Xyloglucan-Endo-Transferase/Hydrolase, which leads to enlargement of the roots (Devos et al. 2005). Auxin and cytokinin signaling can be detected 5 and 3 days following inoculation with *P. brassicae*, respectively, and continues to stimulate growth as the

galls develop (Devos et al. 2006). The source(s) of the increased auxin and cytokinin has not been established conclusively, although Dekhuijzen (1981) reported that cytokinins were present both in the host cytoplasm and pathogen plasmodia. An *Arabidopsis thaliana* L. mutant with respect to genes encoding proteins involved in auxin biosynthesis and metabolism was very susceptible to *P. brassicae* (Jahn et al. 2013); another *A. thaliana* mutant, which overexpressed cytokinin oxidase/dehydrogenase encoding genes, was resistant to *P. brassicae* (Siemens et al. 2006; Schuller et al. 2014). There is also genetic evidence that hormone levels in the plant host are modulated by *P. brassicae* (Schwelm et al. 2015). Nonetheless, knowledge of the exact roles of auxin and cytokinin in the clubroot pathosystem is still limited, and a deeper exploration of plant hormone physiology during pathogenesis may provide insights into clubroot resistance and susceptibility.

1.5.5 Environmental factors affecting clubroot development

Soil properties

Colhoun (1953) reported that clubroot severity was higher in heavy acidic soil than light soil. Karling (1968), however, reported that light, sandy, humus and clay favored the development and severity of clubroot. In another study, the soil types associated with the highest clubroot infection levels were mixtures of soil and sand or clay and sand (Palm and McNew 1956). Soil texture may affect the mobility of *P. brassicae* zoospores (Samuel and Garrett 1945). Poor drainage and waterlogging of soil is also associated with increased clubroot severity (Russell 1859; Somerville 1895), and the disease is significantly reduced in well-drained soil (Dixon and Tilston 2010). Soil texture affects pH and calcium availability, which were also found to influence clubroot severity (Campbell and Greathead 1996). The application of some organic amendments reduced clubroot severity (Niwa et al. 2007) by improving soil texture (Dixon and Tilston 2010).

Clubroot development is favored in slightly acidic soils. As a result, the application of lime or other treatments that increase the soil pH is a well-known clubroot management strategy. The effects of alkaline pH are most pronounced at the early stages of resting spore germination, maturation of primary plasmodia and zoosporangium formation. Many studies have found reduced root hair infection at alkaline pH (Myers and Campbell 1985; Webster and Dixon 1991; Donald and Porter 2004; Rashid et al. 2013). Nonetheless, increasing the soil pH is not always sufficient to provide satisfactory clubroot control under field conditions (Karling 1968; McDonald et al 2004). Gossen et al. (2013) found that a slightly alkaline pH reduced but did not prevent primary and secondary infection or clubroot symptom development under otherwise favorable conditions. Other factors, such as the amount of moisture, the soil inoculum load and the virulence of pathogen strains may influence clubroot severity under field conditions (Dixon 2009; Strelkov and Hwang 2014). The application of calcium has effects similar to higher pH in controlling clubroot (Shinoda 2005; Hwang et al. 2011).

Free water is necessary for the emergence and movement of primary zoospores, and hence clubroot tends to be more severe wet vs. dry soils. This was demonstrated in many early studies. For example, Wellman (1930) found that as soil moisture increases, clubroot develops much more quickly. Similarly, Ayers (1944) reported that clubroot incidence on turnip increased with increased soil moisture. Dixon (1981) showed that clubroot severity increased when moisture was >50% of the soil water holding capacity. A mathematical model proposed by Yang (2004) seeks to explain the relationship between host invasion and soil moisture.

Temperature

Temperature is another important factor in early infection and development of clubroot. Colhoun (1953) found that a combination of alkaline soils and an air temperature of 23℃ represented

favorable conditions for clubroot disease. Even earlier studies showed that temperatures below 20℃ hinder the development of the disease (Chupp 1917), although symptoms may still develop (Monteith 1924; Wellman 1930). Clubroot severity was found to be highest in short-season Brassica crops harvested in July or August, and very low in crops harvested in October when temperatures were low (McDonald and Westerveld 2008). Buczacki (1978) reported that temperature is a very important in the second week after inoculation, because root hair infection reaches its highest level at this time and plasmodia and sporangia are being formed. More recently, Sharma et al. (2011a; 2011b) reported that the development of primary and secondary infection was slow when temperature was <17℃, but increased significantly to a maximum at 23-26℃; infection then began to decrease again as the temperature went up to 30℃. Similar results were obtained by Gossen et al. (2012). Temperature has also been suggested to influence the expression of host resistance genes (Roback and Gabrielson 1988). For example, cauliflower was susceptible at 20℃ under high inoculum densities but was resistant at 15℃.

Nutrients

Calcium has been used for clubroot control for almost a century (Wellman 1930), and has been shown to reduce severity of the disease on many Brassica crops (Klasse 1999; Donald et al. 2002, 2004; Porth et al. 2003; Bhattacharya and Mandal 2006). The application of calcium not only increases soil pH, but also inhibits growth of the pathogen (Webster and Dixon 1991). In Canada, the use of calcium and nitrogen to manage clubroot has been assessed in several studies (McDonald et al. 2004; Tremblay et al. 2005; Abbasi and Lazarovits 2006). Nitrogen fertilizer also has been suggested to reduce clubroot severity (Dixon 2009; Gossen et al. 2014). Previous studies indicated that when nitrate concentration increases to high levels, RNA polymerase sites become saturated with substrate and amino acid moieties are converted to forms that are

inhibitory to *P. brassicae* (Webster 1986). A reduced cofactor NAD(P)H [nicotinamide adenine dinucleotide (phosphate)-reduced] can regulate metabolic processes that convert nitrate to ammonium (Hewitt 1970). These findings suggest that nitrate ions in the rhizosphere were detrimental to *P. brassicae*. A recent study showed that nitrogen can modulate quantitative trait loci for clubroot resistance (Laperche et al. 2017).

Boron also has been found to affect clubroot development. Webster and Dixon (1991b) observed that an increased boron concentration significantly inhibited the development of primary plasmodia, suggesting (Dixon 1991) that boron decreases the rate of sporangial maturation in *P. brassicae*. In addition, boron also appears to strengthen plant cell walls (Loomis and Durst 1992) and stabilize plasma membranes (Cakmak et al. 1995). Craig and Dixon (1993a, b) reported that boron also reduces the ability of *P. brassicae* to cause root hair infection in the field, likely by compromising the ability of *P. brassicae* primary zoospores to penetrate the root hairs. In Canada, a high rate of boron (16 and 32 kg ha^{-1}) could reduce clubroot severity by 25-35% in a muck soil, while it was not associated with any significant reduction in clubroot in a mineral soil (Deora et al. 2013).

1.5.6 Management of clubroot

Crop rotation

Rotation is beneficial for the production of many crops (Cook 2006) because it can interrupt the lifecycle of pathogens and their habitats. In western Canada, the acreage of canola has increased substantially over the past few decades because of its high economic benefits (Canola Council of Canada 2019). Cathcart et al. (2006) reported that a 3-year rotation (canola once every 3 years) is sustainable; however, rotations shorter than 3 years are very common (Hartman 2012). Given that the "half-life" of *P. brassicae* resting spores is ca. 4 years (Wallenhammar 1996; Hwang et

al. 2014), long rotations out of canola and other crucifers are often recommended to manage clubroot. Wallenhammar (1999), for instance, estimated that an interval of 4-6 years away from a Brassica crop might be required to decrease the level of *P. brassicae* inoculum sufficiently. More recently, Peng et al. (2014) recommended a 2-year interval with non-host crops, together with clubroot resistance, in clubroot-infested fields.

Resistance

The planting of clubroot resistant (CR) canola cultivars in appropriate rotations is the most effective, efficient and environmentally friendly way to manage clubroot (Rahman et al. 2014). In Canada, no CR canola cultivars were available until 2009, when the variety "45H29" was introduced to the market; numerous other CR cultivars were introduced soon afterwards by various seed companies (Strelkov and Hwang 2014). Although resistance is proprietary and not in the public domain, there is some evidence suggesting that many CR canola cultivars in Canada derive their resistance from *B. rapa* subsp. *rapifera* (European Clubroot Differential (ECD) 04) or the winter oilseed rape 'Mendel' (Fredua-Agyeman et al. 2018). Unfortunately, since resistance in most cultivars appears to be monogenic, new *P. brassicae* pathotypes have been detected recently in Canada that are highly virulent on 'CR canola' (Strelkov et al. 2016, 2018). Indeed, the loss or erosion of clubroot resistance has been reported previously on a variety of brassica crops, including Chinese cabbage (Hatakeyama et al. 2006) and oilseed rape (Oxley 2007), across many regions. Given the potential for the emergence of new pathotypes of *P. brassicae*, resistance stewardship and the introgression of polygenic resistance should be priorities.

Other management strategies

While genetic resistance and crop rotation are two of the main methods to manage clubroot,

other strategies also exist. The sanitization of field equipment and machinery to remove potentially infested soil is particularly important to slow spread of the disease (Hwang et al. 2014). The application of fungicides or biofungicides also has been studied (Peng et al. 2014), but no products are registered for canola at present. Considering the positive effects of increased soil pH in reducing clubroot severity, several studies have examined the application of lime to manage clubroot in canola (Hwang et al. 2011), and work in this area continues.

1.6 Plant Breeding and QTL Mapping

1.6.1 Plant breeding

Plant breeding aims to increase the yield of crops, develop disease resistance, improve crop quality, and enhance adaptability to environmental stresses. In addition, breeding activities may be carried out to satisfy special market requirements or to meet the needs of special production systems. Following Mendel's work in the 1800s, plant breeding has been linked to an improved understanding of genetics and the heritability of traits. Today, plant breeding involves the manipulation of genetic components, which can be heritable, as well as asexual manipulation (Acquaah 2012).

The phenotype of a plant is determined by the interaction between the genotype (G) and the environment (E). As a consequence, the alteration of G, E or both can vary the phenotype. In plant breeding, the focus is on genetic variability, which can result from genetic recombination, ploidy modification, mutation, and the presence of transposable elements (Acquaah 2012). Desirable variability can be introgressed into a new cultivar by a manipulation of genetics (Humphreys et al. 2003). Selection is a very useful way to identify the most desirable phenotypes and genotypes in a variable population. Selection methods include: mass selection, pure line selection, pedigree selection, bulk selection and single-seed descent.

1.6.2 QTL mapping

A quantitative trait locus (pl. quantitative trait loci, QTL) is a region(s) on a chromosome that is (are) associated with a specific quantitative trait (Tanksley 1993). Quantitative trait loci (QTL) are usually located on different chromosomes. However, a single gene or a cluster of linked genes can control these complex traits (Lander and Schork 2006). QTL mapping, as a kind of molecular breeding method, has drawn intense interest among plant breeders and biologists (Moose and Mumm 2008). Many important traits in plants and animals are complex in nature and are usually controlled by multiple genes. Phenotypes associated with quantitative traits show a continuous distribution and their expression is significantly affected by environmental conditions. Typical quantitative traits include yield, quality, resistance and time to flower. In contrast, qualitative (Mendelian) traits are usually controlled by a single or a few genes, phenotype expression has a discrete distribution, and these traits are not significantly affected by environmental conditions. Typical qualitative traits include flower color and seed shape.

Principles of QTL mapping

Finding and identifying the sites of molecular markers on chromosomes that correlate with quantitative traits is called QTL mapping (Tanksley 1993). In brief, molecular markers and statistical models are used to detect the association between phenotype and genotype of markers (Jamann et al. 2015). To do this, variation in the loci of all of the markers used to genotype the mapping population and the phenotypes of the traits under study are built into a matrix. Correlative statistics is then applied to partition the mapping population into different classes based on the genotype and phenotype data. A marker locus is linked to a QTL controlling the trait if it can partition the mapping population into two or more groups based on significant differences in the phenotypic means. The mean of each group with a tightly linked marker will

be significantly different ($P < 0.05$) from the mean of those without the marker. In contrast, unlinked markers are far from a QTL and hence undergo independent segregation leading to random inheritance (Veeresha et al. 2015). As a result, no significant difference will be detected in the means of the groups with and without the marker.

Development of a mapping population

QTL mapping is dependent on the availability of a mapping population segregating for the desired trait. The main types of mapping population include F_2 families, recombinant inbred lines (RIL), BC (backcross) and DH (doubled haploid) lines. Quantitative traits must be investigated in large populations to minimize the occurrence of false positives (Type I errors) (Visscher et al. 1996). However, due to cost constraints and space limitations for screening large populations, many journals accept for publication purposes QTL studies with a minimum sample size of about 120 to 150 genotypes based on DH and RIL populations and 250 to 300 genotypes for F_2 populations. In addition to populations achieved by crossing of two parents, natural populations are sources to detect QTLs by analyzing linkage disequilibrium (Yu et al. 2008).

Genotyping with molecular markers

QTL mapping requires genotyping of the parents and the mapping population with molecular markers. Molecular markers reveal polymorphisms at the DNA level. Variation in DNA at the molecular level can occur as a result of single nucleotide changes, insertions or deletions (Indels) and variations in the number of tandem repeats (Vignal et al. 2002). Segregation of alleles at any locus follows Mendel's laws of inheritance. Molecular markers can be also be classified as dominant (heterozygotes cannot be distinguished from homozygotes) or co-dominant (heterozygotes can be distinguished from homozygotes). Molecular markers used in the early days of molecular marker studies included bi-allelic dominant markers (random amplified

polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP)) and bi-allelic co-dominant markers (restriction fragment length polymorphism (RFLP) and single-stranded conformation polymorphism (SSCP)). Since the early 2000s, multi-allelic co-dominant markers such as microsatellite or simple sequence repeat (SSR) markers have been the markers of choice, mainly because of the ease of detection of alleles by agarose or polyacrylamide gel electrophoresis and the high number of alleles present at each locus (high heterozygosity values). More recently, single nucleotide polymorphism (SNP) (bi-allelic co-dominant) markers have been used in many genetic studies because of their adaptation to high throughput screening of a large number of loci to provide high-density genetic maps.

Greenhouse and field phenotyping

In addition to the genotype data, QTL mapping also requires phenotype data. As a result, a well-designed greenhouse and (or) field experiments with randomization and replications need to be conducted in which the trait(s) of interest can be assessed. It is also important to carry out field trials at multiple locations to study QTL × Environment interactions.

Linkage map construction

Locus ordering is a very important part of linkage map construction. Methods for locus ordering have been implemented in various mapping packages such MapMaker (Lander et al. 1987) and JoinMap (Stam 1993). A genetic linkage map is a chromosome map that shows the relative positions of markers rather than their physical positions. A genetic map is constructed based on linkage between genes and their crossover value. Based on the position of the first marker on the chromosome, the position of each marker can be calculated in centimorgans (cM) relative to the first marker.

1.6.3 QTL mapping methods

Single marker analysis (SMA)

Single marker analysis (SMA) is the simplest of the QTL mapping methods. It tests the association between phenotype and alleles at each marker locus separately without taking into consideration the relative position of markers (Ronin et al. 1995). It does not require prior construction of a linkage map. The significance of the trait association can be tested using a T-Test, analyses of variance (ANOVA) or linear regression. The major drawbacks of SMA are the inability of markers to detect QTL that are more distant and the underestimation of the effects of QTL.

Simple interval mapping (SIM)

Lander and Botstein (1989) originally developed simple interval mapping (SIM). A problem with SMA is that if a QTL is located between two markers, both markers absorb the partial effect of the QTL. As a consequence, the true position and the true effect of QTL cannot be revealed accurately. Interval mapping analysis examines the associations between adjacent pairs of linked markers to infer the genotype of one locus. As a result, the putative location between two markers can be evaluated for its association with a quantitative phenotypic trait. Therefore, simple interval mapping tests the association between trait values and adjacent pairs of linked markers along chromosomes, simultaneously, instead of analyzing single markers. The construction of a linkage map is a pre-requisite in SIM. The significance of the trait association at every position in the interval is usually tested using the logarithm of odds (LOD) ratios score (critical threshold for most studies is LOD ≥ 3).

Composite interval mapping (CIM)

Composite internal mapping (Zeng 1993; Jiang and Zeng 1995) combines interval mapping with

multiple regression (Veeresha et al. 2015). CIM analyzes the target locus together with reference markers at multiple analysis points across each inter-marker interval. The reference markers serve as covariates to control for other QTL and to reduce the residual variance. As a result, CIM is more precise and effective than SMA and SIM. It is able to separate the target QTL from other linked QTLs and so it is especially useful when QTLs are linked. Programs used for CIM mapping include QTL Cartographer (Basten et al. 2001) and Map Manager QTL (Manly et al. 2001).

Multiple interval mapping (MIM)

Multiple interval mapping (MIM) uses multiple marker intervals simultaneously to infer the location of multiple QTLs. QTL detection is controlled directly in a model for QTL mapping. MIM improves the precision and power of QTL mapping and enables the estimation of epistasis between QTL, genotypic values and heritabilities (Kao et al. 1999).

1.6.4 QTL mapping in canola breeding

Different types of markers (RFLP, SSR, SNP etc.) have been used to construct genetic maps in the Brassicas for over two decades (Ecke et al. 1995; Zhao et al. 2005; Delourme et al. 2013). QTLs have been identified for agronomic traits such as oil content (Burns et al. 2003; Wang et al. 2013; Jiang et al. 2014) and protein content (Zhao et al. 2006; Würschum et al. 2012). With respect to QTL mapping of resistance to canola diseases, Larkan et al. (2016) evaluated two DH populations and detected three quantitative blackleg (*L. maculans*) resistance loci in each population. Wu et al. (2013) mapped 10 QTL for stem rot (*S. sclerotiorum*) resistance at the mature plant stage and three QTL for leaf resistance at the seedling stage on nine linkage groups. Makers for QTL linked to agronomic traits and disease resistance can be applied in marker-assisted selection (MAS) to facilitate and improve canola breeding.

1.6.5 Clubroot resistance breeding and genetics

Clubroot resistance has been reported in *B. rapa*, *B. napus*, *B. oleracea*, *B. nigra*, but not in *B. juncea* or *B. carinata* (Diederichsen et al. 2009; Fredua-Agyeman et al. 2019). Most of the resistance appears to be race or pathotype-specific, except for some resistance in *B. oleracea* which was found to be race-independent (Diederichsen et al. 2009). In *B. rapa*, resistance is mainly from Chinese cabbage (*B. rapa* subsp. *pekinensis*), which is widely grown in east Asia (Piao et al. 2009), and from oilseed turnip (*B. rapa* subsp. *rapa*), which is a spring variety grown in regions with a short growing season such as Canada and Scandinavia (Piao et al. 2009).

Genetic studies of *B. rapa* have identified several genes associated with clubroot resistance, including *CRa* (Matsumoto et al. 1998), *CRb* (Piao et al. 2004) and Crr1 (Suwabe et al. 2003, 2006). *Brassica oleracea* is less commonly used than *B. rapa* in resistance breeding. The cabbage 'Badger Shipper' was the first CR cultivar of *B. oleracea* introduced to North America, although the resistance did not prove to be durable (Crute et al. 1980). Broccoli (*B. oleracea* var. *italica*) is another good source of clubroot resistance (Baggett 1983; Baggett and Kean 1985). In contrast with *B. rapa*, however, resistance in *B. oleracea* is complex and appears to be under the control of race-specific and race-independent QTL (Diederichsen et al. 2009). *Brassica napus* is an amphidiploid obtained from *B. rapa* and *B. oleracea* and, therefore, its resistance is derived from *B. rapa* (A genome) and *B. oleracea* (C genome). The CR winter oilseed rape 'Mendel' was derived from ECD 04, which possesses three major clubroot resistance genes (AA+BB+CC), and ECD 15, a CR kale (*B. oleracea* var. *sabellica*). 'Mendel' became available on the European market in 2000. Diederichsen et al. (2006) reported that there is one major resistance gene and two recessive genes in this cultivar, indicating the loss of two major clubroot resistance genes during the breeding process. As noted earlier in this review,

'Mendel' appears to be the source of clubroot resistance in most Canadian CR canola (Fredua-Agyeman et al. 2018). Werner et al. (2008) reported 19 race-specific QTL in a DH population (ECD 04 × one cabbage landrace) from which the *B. oleracea* resistance should have been race-independent.

1.7 Research Objectives and Hypotheses

This Ph.D. project aimed to contribute to knowledge of clubroot pathogenesis as well as to the discovery of alleles for clubroot resistance. There were three specific objectives: (i) to study the effects of primary infection by virulent *P. brassicae* pathotypes on subsequent host resistance to avirulent pathotypes, (ii) to identify pathogen genes differentially expressed during pathogenesis in compatible and incompatible interactions, and (iii) to identify and introgress clubroot resistance from a resistant *B. rapa* host (ECD 02) into two susceptible *B. rapa* genotypes (CDCN 061 and CDCN 155). I hypothesized that (i) virulent isolates of *P. brassicae* will compromise host resistance during primary infection, even at low resting spore concentrations, (ii) expression of certain genes by *P. brassicae* will differ in compatible and incompatible interactions, and (iii) clubroot resistance from ECD 02 can be introgressed into CDCN 061 and CDCN 155 by making crosses.

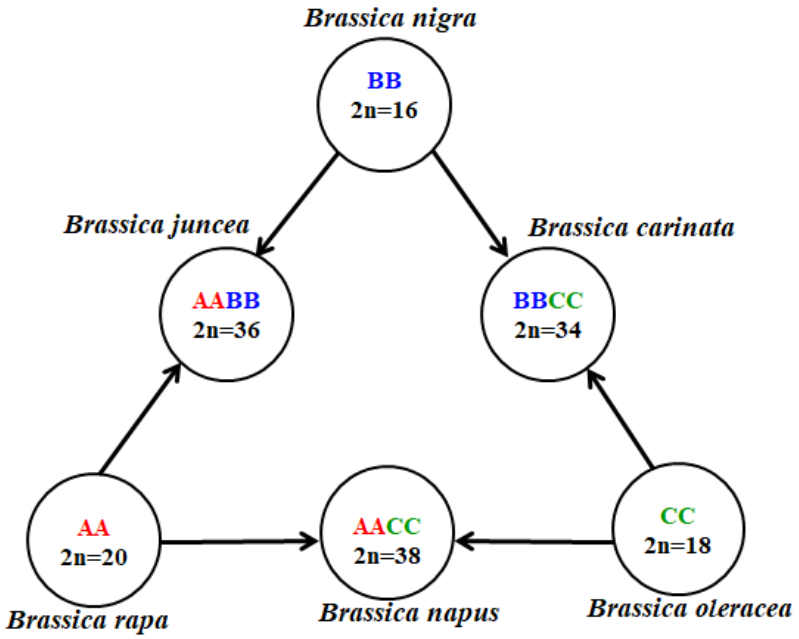

Figure 1.1. The 'triangle of U', showing genome relationships between the six Brassica species. *Brassica nigra*, *B. rapa* and *B. oleracea* are diploid species, while *B. juncea, B. carinata* and *B. napus* are amphidiploid species.

Chapter 2: Suppression of Canola (*Brassica napus*) Resistance by Virulent Isolates of *Plasmodiophora brassicae* (clubroot) During Primary Infection[1]

2.1 Introduction

The life cycle of *Plasmodiophora brassicae* Woronin, the cause of clubroot of canola (*Brassica napus* L.) and other crucifers, includes three distinct stages: resting spore survival in the soil, primary (root hair) infection, and secondary (cortical) infection (Hwang et al. 2012). Under favorable conditions, which include high soil moisture, moderate temperature and the presence of root exudates, the long-lived resting spores of the pathogen are stimulated to germinate and produce primary zoospores (Dixon 2014). The primary zoospores invade the root hairs of potential hosts and form primary plasmodia, which subsequently undergo nuclear divisions and cleavage into zoosporangia that contain 4 -16 secondary zoospores each (Kageyama and Asano 2009; Howard et al. 2010). These secondary zoospores are released into the rhizosphere and re-infect the host, producing secondary plasmodia within the cortical tissues of the main roots. Proliferation of the secondary plasmodia results in hypertrophy and hyperplasia of the infected cortical cells and the characteristic clubbing of the roots (Kageyama and Asano 2009; Howard et al. 2010). Secondary plasmodia eventually are cleaved into large numbers of resting spores, which are released back into the soil as the galls degrade, serving as inoculum to start new cycles of infection (Hwang et al. 2012).

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In the life cycle of *P. brassicae*, primary zoospores are produced before secondary zoospores, but both can sometimes exist simultaneously in root hairs or epidermal cells. Varying lengths of time have been reported for the onset of primary infection and the development of secondary infection. Dobson and Gabrielson (1983) and Feng et al. (2013a) detected primary zoospores in the root hairs of Chinese cabbage (*Brassica rapa* L. var. *pekinensis*) and canola, respectively, 12 - 24 h after inoculation, while secondary zoospores were observed 2 - 3 days after inoculation (dai). Other researchers, however, have reported the observation of both primary and secondary infections much later after inoculation. Primary and secondary infections were observed after 3 - 4 days and 5 - 8 days, respectively, when turnip (*Brassica rapa* L. var. *rapifera*) root hairs and *P. brassicae* were cultured together under axenic conditions (Kageyama and Asano 2009). Devos et al. (2005) and Agarwal et al. (2011) observed primary and secondary infection between 4 - 7 dai and 10 - 19 dai, respectively, when Chinese cabbage and Arabidopsis were grown in soil. Therefore, the onset of primary and secondary infection varies with different host genotypes, the virulence of the *P. brassicae* isolates used, as well as their interactions with environmental factors such as temperature, humidity, pH and nutrients.

Primary infection by *P. brassicae* occurs in both host and non-host species (MacFarlane 1952; Ludwig-Müller et al. 1999; Friberg et al. 2006; Feng et al. 2012). Secondary infection of non-host species, however, rarely leads to the development of typical clubroot symptoms (MacFarlane 1952; Ludwig-Müller et al. 1999; Hwang et al. 2015). It is difficult to estimate the contribution of primary and secondary zoospores to clubroot development, since these spore types cannot be distinguished from each other based on morphology or general biological function (Kageyama and Asano 2009). Nonetheless, in spite of the inextricable similarities in morphology, the two phases of the life cycle are distinct. Dobson and Gabrielson (1983)

demonstrated by controlling soil water matric potential that the observation of secondary zoospores coincided with the initiation of cortical infection. The two types of zoospores also seem to have different roles in pathogenesis, based on the number of genes that are upregulated or downregulated in resistant cultivars during primary and secondary infection (Fei et al. 2016; Feng et al. 2013b).

The aim of this study was to test the hypothesis that when low resting spore concentrations of different virulent *P. brassicae* isolates are inoculated on seedlings of resistant canola cultivars for a short time, they infect the roots and suppress the resistance of the host to avirulent isolates. Challenging seedlings of the same cultivar independently with virulent and avirulent isolates of the pathogen permitted the comparison of the experimental treatments to the positive and negative controls. Lastly, the inoculation of resistant seedlings first with virulent isolates followed by avirulent isolates provides a window into the possible damage that the emergence of even low concentrations of new *P. brassicae* pathotypes could cause canola and other *Brassica.*

2.2 Materials and methods

2.2.1 Plant materials and *P. brassicae* isolates

Three clubroot resistant (CR) canola cultivars (‘‘45H29’’, ‘L135C’ and ‘L241C’) were evaluated for their reaction to three pairs of avirulent/virulent isolates of *P. brassicae* obtained from infected roots collected before and after the introduction of the clubroot resistance trait in Alberta, Canada. The ‘avirulent’ or ‘virulent’ designations refer to the capacity of the isolates to cause disease on CR canola. The avirulent isolates (‘SACAN-ss3’, ‘SACAN-ss1’ and ‘ORCA-ss4’) were single-spores and classified as pathotypes 2, 3 and 5, respectively, on the differentials of Williams (1966) (Xue et al. 2008). The virulent isolates (F183-14, CDCN#4 and F-359-13)

represent field isolates (collections of resting spores derived from single root galls) that were able to overcome the resistance(s) in "45H29", 'L135C' and 'L241C' (Strelkov et al. 2016; 2018). They were classified as pathotypes 2, 3, and 5 on the differentials of Williams (1966), but will hereafter be referred to as pathotypes 2*, 3* and 5* to denote their increased virulence on CR canola. All six single-spore and field isolates were maintained as resting spores in frozen root galls on the universal clubroot-susceptible host Chinese cabbage (*B. rapa* var. *pekinensis*) 'Granaat'.

2.2.2 Inoculum preparation

Frozen galls of each pathotype were homogenized separately in sterile distilled water in a Waring LB10G blender (Cole-Parmer) as described by Fredua-Agyeman et al. (2018). The spore suspension was collected by passing the slurry through eight layers of cheesecloth into a conical flask. The concentrations of resting spores were quantified with a haemocytometer (VWR, Mississauga, ON, Canada) and adjusted with sterile distilled water. Two spore suspensions of each of the virulent pathotypes 2*, 3* and 5* were prepared, at concentrations of 1×10^3 spores/mL (low) and 1×10^7 spores/mL (high). A single concentration (1×10^7 spores/mL (high)) of each of the avirulent pathotypes 2, 3 and 5 was prepared.

2.2.3 Clubroot tests

To carry out clubroot tests, about 2000 - 2400 seedlings each of "45H29", 'L135C' and 'L241C' were produced per experiment by pre-germinating seeds on moistened sterile filter paper in Petri dishes (100 mm × 15 mm) for 7 days at room temperature and a 12h photoperiod. Each experiment consisted of five treatments comprising nine combinations ("45H29", 'L135C' and 'L241C' × 2*/2, 3*/3 and 5*/5) (Table 2.1).

The experimental treatments (ETs) comprised roots of seedlings of each cultivar in Petri dishes (100 × 15 mm) incubated for 2 days with low concentrations (50 mL; 1×10^3 spores/mL × 30 seedlings/Petri dish) of resting spores of each of the virulent pathotypes, followed by washing off the inoculum from the roots and a subsequent re-inoculation by dipping the washed roots into high concentrations (1×10^7 spores/mL) of resting spores of the corresponding avirulent pathotype. The seedlings were transplanted into 8 × 4 flat trays, cell size 7 × 6 × 6 cm, filled with Sunshine Mix #4 Aggregate Plus growing mix (Sungro Horticulture, Seba Beach, Alberta, Canada).

Two positive controls were used for each of the three cultivars. The first (PC1) comprised seedlings incubated for 2 days with low concentrations of each virulent pathotype followed by washing off the inoculum and a second inoculation with high concentrations of the corresponding virulent pathotypes. The second positive control (PC2) comprised seedlings inoculated only with high concentrations of the virulent pathotypes. The seedlings were transplanted into Sunshine Mix #4 Aggregate Plus growing mix as described above. Similarly, two negative controls were used in each experiment, which were comprised of seedlings inoculated only with high concentrations of the avirulent pathotypes (NC1) or only with low concentrations of the virulent pathotypes (NC2) before being transplanted into the growing mix.

The plants were kept in a greenhouse maintained at 20-25/15-18 ^{o}C day/night, 16 h photoperiod, and watered daily with slightly acidified water (pH ≈ 6, adjusted with HCl). Fertilizer (N: P: K = 20: 20: 20) was applied once a week 3 weeks after transplanting. The inoculation experiments were arranged in a randomized complete block design with the different isolates as the main plots and the different cultivars as the subplots. Each inoculation treatment was carried out in triplicate with each repetition consisting of 120 - 160 seedlings. The trays

were randomized twice in the 2nd and the 4th weeks after the seedlings were transplanted into potting mix and each experiment was repeated three times.

2.2.4 Sampling and disease assessment

Root samples were collected 1, 3, 5, 7, 9, 11, 14, 17, 21, 28 and 35 dai for the five treatment combinations for each cultivar. Roots of three inoculated plants from each treatment were selected randomly, uprooted gently, and washed carefully in tubs containing water. The root samples (approx. 4455 = 11 time points × 5 treatments × 3 cultivar × 3 pathotypes × 3 plants × 3 replicates) were stored at -20°C until DNA extractions were carried out to determine the relative quantity (RQ) of pathogen biomass to the host by quantitative PCR (qPCR) analysis (see below).

The remaining plants (actual sample size 25 - 97 plants/treatment/replication; not all seedlings survived transplantation) were dug out, washed with water and rated for clubroot symptom development 6 weeks after inoculation. Clubroot symptoms were rated on a scale of 0 - 3 according to Kuginuki et al. (1999), where 0 = no galls; 1 = a few small or bead-sized galls on < 1/3 of the roots; 2 = medium galls on 1/3-2/3 of the roots, and 3 = large galls on > 2/3 of the roots. A disease severity index (DSI, 0 - 100%) was calculated for each replication using the formula of Horiuchi and Hori (1980) as modified by Strelkov et al. (2006):

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

Where n is the number of plants in one class and N is the total number of plants in one biological replicate. The mean DSI for each treatment and for each experiment was calculated by averaging the DSIs of experiments 1, 2 and 3.

Disease incidence (DI) was calculated with the formula: $\text{DI (\%)} = \frac{m}{N} \times 100$; where m is the number of infected plants and N is the total number of plants in one biological replicate. The

mean for each treatment and for each experiment was calculated by averaging the DIs of the three replicated experiments.

2.2.5 DNA extraction

Total genomic DNA (canola + *P. brassicae*) was extracted from root galls of each of the three replicates for each treatment using the cetyltrimethyl ammonium bromide (CTAB) method (Sambrook and Russell 2001). The DNA concentration was quantified using a ND-2000c spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and the final concentration of each sample was adjusted to 20 ng DNA/µL.

2.2.6 Real-time PCR and relative quantification of *P. brassicae* biomass

The comparative Ct or the Delta-Delta Ct (ΔΔCt) qPCR method (Livak and Schittgen 2001, Schittgen and Livak 2008) was used to determine the RQs of *P. brassicae* DNA and canola DNA. The *P. brassicae* specific primers (TC1F: 5′-GTGGTCGAACTTCATTAAATTTGGGCTCTT-3′ and RTPbR1a: 5′-TCAGCACCGTTTCCGGCTGCTAAGGC-3′) (Cao et al. 2014) and canola specific β-actin (*BnAct2*) primer (F: 5′-ACGAGCTACCTGACGGACAAG-3′; R: 5′-GAGCGACGGCTG-GAAGAGTA-3′) (Yang et al. 2009) were used to confirm the similarity of the PCR efficiency of the pathogen and the host housekeeping genes, as well as reactions for the determination of the RQs of *P. brassicae* and canola DNA from the roots harvested over the time course. The specificity of the primers was confirmed by melt curve analysis and also on 2% agarose gels.

To validate the efficiency of the PCR, two independent standard curves were generated by plotting cycle numbers against each of *P. brassicae* DNA diluted to final concentrations of 40 ng/µL, 4 ng/µL, 0.4 ng/µL, 40 pg/µL, 4 pg/µL and 0.4 pg/µL and non-infected canola root DNA

diluted to final concentrations of 100 ng/µL, 10 ng/µL, 1 ng/µL, 0.1 ng/µL and 10 pg/µL (i.e., a 10-fold dilution per step). The ΔCt (Ct $_{P.\ brassicae\ \text{DNA}}$-Ct $_{\text{canola DNA}}$) was calculated for each dilution. The suitability of the ΔΔCt method was then determined from the absolute value of the slope of the plot of ΔCt versus log DNA dilutions (Livak and Schittgen 2001).

The qPCRs of the harvested samples were run in triplicates and also within each replicate three DNA repeats were used. SYBR Green was used as the fluorescent DNA binding dye on a QuantStudio 6 Flex Real-Time PCR System (Fisher Canada, Ottawa, ON). The qPCR conditions consisted of initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. After each run, a dissociation curve was obtained by heating the samples from 60°C to 95°C.

The ΔCt and ΔΔCt were calculated using the formulae of Livak and Schittgen (2001) and Schittgen and Livak (2008) as follows: $\Delta Ct_{\ 1} = Ct_{\ P.\ brassicae\ \text{gene}} - Ct_{\ \text{non-infected canola gene}}$, $\Delta Ct_{\ 2} = Ct_{\ \text{canola } \beta\text{-actin gene}} - Ct_{\ \text{non-infected canola gene}}$, and $\Delta\Delta Ct = \Delta Ct_{\ 1} - \Delta Ct_{\ 2}$, where the threshold cycle (Ct) is the cycle number when the fluorescence of the sample exceeds the background cycle. Non-infected canola gene was used as a calibrator to normalize two different qPCR experiments. The quantity of *P. brassicae* relative to the amount of plant tissue and normalized by the calibrator was then calculated as $2^{-\Delta\Delta Ct}$.

2.2.7 Statistical analyses

Data collected were analyzed by analysis of variance (ANOVA) using the General Linear Model (GLM) in R software (R Development Core Team 2015). The GLM formula used was $y_{ij} = \mu + \tau_i + \varepsilon_{ij}$; where i indexed experimental treatments in each combination and j indexed observations within the i^{th} group. Comparisons among means of DSI ± standard error and DI ±

standard error were conducted at $P = 0.05$ with Duncan's test (Steel and Torrie 1960). Statistical graphs in Excel were converted into boxes and whiskers.

2.3 Results

2.3.1 Clubroot disease severity and incidence

The clubroot severity and incidence data (mean ± standard error and range of three repeated experiments) of nine treatment combinations involving three pairs of virulent/avirulent pathotypes (i.e. 2*/2, 3*/3 and 5*/5) × three canola cultivars (i.e. ''45H29'', 'L135C' and 'L241C') are presented in Tables 2.1 and 2.2, respectively, and also as box and whisker plots in Figures 2.1 and 2.2.

The inoculation of seedlings with high concentrations (1×10^7 spores/mL) of pathotypes 2, 3 and 5 (NC1) resulted in Dis of 7.9 ± 2.1 to 17.9 ± 7.2% on ''45H29'', 7.0 ± 5.2 to 11.9 ± 9.4% on 'L135C' and 5.8 ± 3.4 to 8.4 ± 6.6% on 'L241C'. This confirms that the isolates representing pathotypes 2, 3 and 5 were avirulent on these three cultivars. In contrast, inoculation with high (1×10^7 spores/mL) concentrations of pathotypes 2*, 3* and 5* (PC1 and PC2) resulted in significantly higher Dis of 94.2 ± 2.3 to 100.0 ± 0.0% for the three cultivars. This confirms that pathotypes 2*, 3* and 5* were virulent on the three cultivars.

Inoculation with low (1×10^3 spores/mL) concentrations of pathotypes 2* and 5* (NC2) also resulted in significantly higher Dis (38.2 ± 11.2% in ''45H29'', 30.0 ± 5.6% on 'L135C' and 58.2 ± 21.1% on 'L241C' for 2*; 31.4 ± 14.1% in ''45H29'', 24.9 ± 5.5% on 'L135C' and 40.0 ± 19.3% on 'L241C' for 5*) (Table 2.2). Inoculation with a low concentration of the third virulent isolate representing pathotype 3*, however, resulted in Dis (3.4 ± 1.0%, 2.9 ± 2.0% and 2.5 ± 1.5% on '45H29', 'L135C' and 'L241C') similar to those obtained with high concentrations of

the avirulent pathotypes (Table 2.2). Therefore, inoculation with 1×10^3 spores/mL of pathotypes 2* and 5* caused clubroot disease on ‘‘45H29’’, ‘L135C’ and ‘L241C’ (in six of six treatment combinations), while inoculation with low concentrations of 3* did not cause any significant clubroot symptoms on the three cultivars (three of three treatment combinations).

The mean DSI (mean ± standard error) values for seedlings inoculated with the 2*/2 pathotype combination (low concentration of pathotype 2* + high concentration of pathotype 2) were 37.9 ± 8.4, 32.8 ± 6.9 and 43.1 ± 15.0% for ‘‘45H29’’, ‘L135C’ and ‘L241C’, respectively (Table 2.1). Following inoculation with the 3*/3 and 5*/5 pathotype combinations, the DSI values were 25.9 ± 5.6, 23.3 ± 8.7 and 27.0 ± 8.9 and 37.6 ± 3.7, and 34.8 ± 3.7 and 44.0 ±13.7%, respectively, for ‘‘45H29’’, ‘L135C’ and ‘L241C’ (Table 2.1). In the case of negative control treatment 1 (NC1; seedlings inoculated only with a high concentration of the avirulent pathotypes 2, 3 or 5), the DSI ranged from 1.9 ± 1.1 to 10.2 ± 4.0%, 2.7 ± 2.1 to 12.0 ± 3.8% and 3.0 ± 2.4 to 6.9 ± 1.6%, respectively, on ‘‘45H29’’, ‘L135C’ and ‘L241C’ (Table 2.1). Thus, the DSI values were significantly (P = 0.05) higher in all nine ETs compared with the NC1 in all pathotype-cultivar combinations.

The DSI values were significantly higher in seven of the nine ET combinations compared with the negative control treatment 2 (NC2) in which seedlings were inoculated only with low concentrations of the virulent pathotypes 2*, 3* and 5*. These seven ET comprised all six 2*/2, 3*/3 and 5*/5 × ‘‘45H29’’ and ‘L135C’ combinations, with DSI values which ranged from 23.3 ± 8.7 to 37.9 ± 8.4% for the Ets and to 2.0 ± 1.7 to 21.9 ± 8.0% for NC2 (Table 2.1). The seventh pathotype-cultivar combination which showed significantly higher (P = 0.05) DSI values in the ET (27.0 ± 8.9%) compared with NC2 (1.3 ± 0.9%) was 3*/3 × ‘L241C’. The 2*/2 and 5*/5 ×

'L241C' combinations did not show any significant difference ($P = 0.05$) in DSI values in the ET (43.1 ± 15.0 to 44.0 ± 13.7%) vs. NC2 (30.3 ± 14.9 to 39.3 ± 17.3%) (Table 2.1).

There was no significant difference in the DSI values of the positive control treatment 1 (PC1) (seedlings inoculated with low concentrations of pathotypes 2*, 3* and 5* + high concentrations of pathotypes 2*, 3* and 5* two days later) (88.0 ± 4.3 to 96.4 ± 0.2%) and positive control treatment 2 (PC2) (seedlings inoculated with high concentrations of pathotypes 2*, 3* and 5*) (89.9 ± 2.8 to 100.0 ± 0.0%). As was expected, the DSI were significantly higher in all nine PC1 and PC2 compared with the Ets (23.3 ± 8.7 to 44.0 ± 13.7%), NC1s (1.9 ± 1.1 to 12.0 ± 3.8%) and NC2s (1.3 ± 0.9 to 39.3 ± 17.3%) (Table 2.1).

In the case of mean DI (mean ± standard error), the values of the Ets ranged from 40.3 ± 17.3 to 65.4 ± 20.3% compared with NC1 (5.8 ± 3.4 to 17.9 ± 7.2%) for the nine treatment combinations (2*/2, 3*/2 and 5*/5× ''45H29'', 'L135C' and 'L241C') (Table 2.2). Thus, the DI values (similar to the DSI trend) were significantly ($P = 0.05$) higher in all nine Ets compared with the NC1 in all pathotype-cultivar combinations.

Furthermore, six of the nine treatments showed significant differences in DI values between the ET and NC2 (Table 2.2). The DI values of the six pathotype-cultivar combinations (3*/3 and 5*/5 × ''45H29''; 2*/2, 3*/3 and 5*/5 × 'L135C'; 3*/3 × 'L241C') for the ETs ranged from 40.3 ± 17.3 to 57.9 ± 8.7% compared with 2.5 ± 1.5 to 31.4± 14.1% for NC2. Similar to the DSI, the DI values of the 2*/2 and 5*/5 × 'L241C' combinations did not show any significant difference ($P = 0.05$) in the ET (55.6 ± 12.7 to 65.4 ± 20.3%) compared with the NC2 (40.0 ± 19.3 to 58.2 ± 21.1%). In addition, the 2*/2 × ''45H29'' combination did not show any

significant difference (P = 0.05) in the ET (58.8 ± 14.0 %) compared with NC2 (38.2 ± 11.2%) (Table 2.2).

Similarly, the DI values of PC1 (97.1 ± 2.9 to 100.0%) and PC2 (94.2 ± 2.3 to 100.0%) were significantly greater than that of the ETs (40.3 ± 17.3 to 65.4 ± 20.3%), NC1s (5.8 ± 3.4 to 17.9 ± 7.2%) and NC2s (2.5 ± 1.5 to 58.2 ± 21.1%) (Table 2.2).

Inoculation with low concentrations of pathotypes 2*, 3* and 5* followed two days later by inoculation with high concentrations of pathotypes 2, 3 and 5 or 2*, 3* and 5*, respectively, caused significant levels of clubroot (see above for values of DSI and DI). Thus, pathotypes 2* and 5* even at low concentrations caused significant clubroot on ‘‘45H29’’, ‘L135C’ and ‘L241C’, but the effect was elevated or magnified when the cultivars were subsequently inoculated with avirulent or virulent isolates. In contrast, pathotype 3* was virulent on the three CR canola cultivars at high concentrations, but at low concentrations inoculation with 3* needed to be followed by a high concentration of 3 or 3* to cause significant disease. Lastly, there were no significant differences (P = 0.05) in the DSI and DI obtained for the different canola cultivars following inoculation with the same isolates (Tables 2.1 and 2.2).

2.3.2 Efficiency and specificity of primers for qPCR assay

The plot of Ct values vs. log serially diluted DNA of *P. brassicae* and genomic DNA of canola confirmed the linearity of amplification over the dynamic range. The slope of the calibration curves was -3.18 (R^2 = 0.999) for *P. brassicae* (Figure 2.3) and -3.21 (R^2 = 0.993) for canola (Figure 2.4), which were very close to the theoretical slope of -3.32 required for qPCR assays with 100% efficiency. The qPCR assays generated a single peak in melting curve analysis which

indicated the specificity of the target. In addition, no amplified product was obtained when pure canola DNA was subjected to PCR with the *P. brassicae* primers.

2.3.3 Quantification of *P. brassicae* DNA relative to canola DNA by qPCR

In the qPCR assay, the RQs ($2^{-\Delta\Delta Ct}$ mean ± standard error of three repeated experiments) of *P. brassicae* biomass to canola ranged from 0.1 ± 0.0 to 34.0 ± 12.3, 0.6 ± 0.3 to 9.2 ± 3.1 and 0.3 ± 0.1 to 16.7 ± 10.4 for the 2*/2, 3*/3 and 5*/5 × ‘‘45H29’’combinations, respectively, for the root samples taken from 1 to 28 dai (Table 2.3). The RQs of *P. brassicae* biomass to canola for the 2*/2, 3*/3 and 5*/5 × ‘L135C’ combinations ranged from 0.1 ± 0.0 to 14.7 ± 7.5, 0.3 ± 0.2 to 26.5 ± 5.2 and 0.3 ± 0.2 to 20.9 ± 0.3, respectively, for the root samples taken over the same period (1 to 28 dai). In the case of 2*/2, 3*/3 and 5*/5 × ‘L241C’, the RQs for the root samples ranged from 0.2 ± 0.1 to 13.2 ± 6.8, 0.3 ± 0.1 to 18.7 ± 1.8 and 0.4 ± 0.2 to 83.7 ± 75.6, respectively. In general, the RQs for all nine combinations (2*/2, 3*/3 and 5*/5 × ‘‘45H29’’, ‘L135C’ and ‘L241C’) were not significantly different ($P = 0.05$) from each other until 35 dai (Figure 2.5). Similarly, the RQs for NC1 and NC2 from 1 to 28 dai were not generally significantly ($P = 0.05$) different from the RQs for the ET over the same period (Figure 2.5). The RQs of *P. brassicae* biomass to canola for NC1 and NC2 in the 2*/2, 3*/3 and 5*/5 × ‘L135C’ and ‘L241C’ combinations (range 0.0 ± 0.0 to 31.2 ± 15.3) were in general only significantly different from the ET at 35 dai (range 6.3 ± 4.1 to 106.8 ± 67.7).

In contrast, the RQs of *P. brassicae* biomass to canola for the two positive controls (PC1 and PC2) were generally significantly greater ($P = 0.05$) at 17 to 35 dai than the RQs of the respective ET, NC1 and NC2 over the same period (Figure 2.5). In the 2*/2 × ‘‘45H29’’ treatments, the RQs following inoculation with low pathotype 2*/high pathotype 2* (PC1) at 21 to 35 dai ranged from 36.5 ± 27.9 to 177.7 ± 76.9, while for the inoculation with high pathotype

2* (PC2) they ranged from 45.1 ± 16.0 to 580.3 ± 88.0. For the low pathotype 2*/high pathotype 2 (ET) combination, the RQs ranged from 3.0 ± 0.4 to 98.1 ± 66.6, compared with a range of 0.5 ± 0.1 to 0.9 ± 0.1 for high pathotype 2 (NC1) and 0.0 ± 0.0 to 0.4 ± 0.2 for low pathotype 2* (NC2). In the case of 3*/3 × ‘‘45H29’’, the RQs following inoculation with low pathotype 3*/high pathotype 3* (PC1) and high pathotype 3* (PC2) ranged from 25.0 ± 21.3 to 236.2 ± 178.0 and 167.0 ± 79.9 to 798.5 ± 119.2, respectively, compared with an RQ range of 6.8 ± 2.4 to 82.2 ± 78.1 for low pathotype 3*/high pathotype 3 (ET), 0.1 ± 0.0 to 0.9 ± 0.3 for high pathotype 3 (NC1), and 0.0 ± 0.0 to 0.4 ± 0.2 for low pathotype 3* (NC2). The RQs for the 5*/5 × ‘‘45H29’’ treatments ranged from 1.3 ± 0.7 to 160.8 ± 114.8 for low pathotype 5*/high pathotype 5* (PC1) and 93.7 ± 17.1 to 284.7 ± 49.4 for high pathotype 5* (PC2) at 21 to 35 dai, compared with RQs of 3.7 ± 1.4 to 23.2 ± 21.0 for low pathotype 5*/high pathotype 5 (ET), 0.7 ± 0.0 to 4.5 ± 2.7 for high pathotype 5 (NC1), and 0.0 ± 0.0 to 0.3 ± 0.1 for low pathotype 5* (NC2).

The RQs of *P. brassicae* biomass to canola for the 2*/2 × ‘L135C’ combination at 21 to 35 dai ranged from 10.2 ± 4.8 to 162.2 ± 23.5 for the low pathotype 2*/high pathotype 2* inoculation (PC1) and 168.9 ± 69.8 to 563.5 ± 290.3 for the high pathotype 2* inoculation (PC2), compared with RQs of 5.4 ± 1.5 to 105.3 ± 50.0 for low pathotype 2*/high pathotype 2 (ET), 1.0 ± 0.3 to 1.4 ± 0.9 for high pathotype 2 (NC1), and 0.0 ± 0.0 to 1.3 ± 1.3 for low pathotype 2* (NC2). For the 3*/3 × ‘‘45H29’’ combination, the RQs for the low pathotype 3*/high pathotype 3* inoculation (PC1) and the high pathotype 3* (PC2) inoculation ranged from 47.0 ± 37.6 to 121.6 ± 51.1 and 53.6 ± 26.2 to 492.6 ± 174.6, respectively, compared with 12.9 ± 5.3 to 56.0 ± 52.1 for low pathotype 3*/high pathotype 3 (ET), 0.1 ± 0.0 to 0.8 ± 0.1 for high pathotype 3 (NC1), and 0.1 ± 0.0 to 1.1 ± 1.1 for low pathotype 3* (NC2). The RQs for the 5*/5 × ‘‘45H29’’ combination ranged from 76.5 ± 73.3 to 143.9 ± 129.0 for the low pathotype 5*/high pathotype

5* inoculation (PC1) and from 40.3 ± 4.5 to 273.9 ± 40.3 for the high pathotype 5* inoculation (PC2), compared with 11.1 ± 3.5 to 23.2 ± 21.5 for low pathotype 5*/high pathotype 5 (ET), 1.7 ± 0.8 to 3.2 ± 1.2 for high pathotype 5 (NC1), and 0.0 ± 0.0 to 1.2 ± 0.3 for low pathotype 5* (NC2).

In the case of the 2*/2, 3*/3 and 5*/5 × 'L241C' treatment combinations, the RQs of *P. brassicae* biomass to canola for PC1 ranged from 10.1 ± 2.7 to 305.2 ± 163.4, while for PC2 they ranged from 95.0 ± 36.2 to 944.3 ± 238.9, compared with RQs of 4.3 ± 1.0 to 106.8 ± 67.7 for the ET, 0.8 ± 0.4 to 4.1 ± 2.2 for NC1, and 0.0 ± 0.0 to 3.3 ± 3.2 for NC2.

Overall, the qPCR data showed that 28 to 35 days was the optimum period at which there was sufficient *P. brassicae* biomass in the ET to be detected by the comparative C_t qPCR method. Nonetheless, *P. brassicae* biomass could be detected at 17 to 21 dai following inoculation of the seedlings with virulent pathotypes (PC1 and PC2). In contrast, *P. brassicae* could not be detected even at 35 dai following inoculation with low concentrations of virulent isolates (NC1) or high concentrations of avirulent isolates (NC2).

2.4 Discussion

The effects of low inoculum concentrations of virulent *P. brassicae* pathotypes on the resistance of three clubroot resistant canola cultivars ('45H29', 'L135C' and 'L241C') and the accumulation of pathogen biomass were studied. While classified as clubroot resistant, the three cultivars were not completely immune to the pathogen and developed mild symptoms of disease when inoculated with high concentrations of the avirulent pathotypes. Nonetheless, when the cultivars were first exposed to a low concentration of virulent inoculum prior to exposure to a high concentration of the avirulent inoculum, the clubroot incidence (DI) and severity (DSI)

were significantly greater than when they were exposed only to the high concentration of avirulent inoculum (NC1) or only to the low concentration of virulent inoculum (NC2). This was observed across all three cultivars and virulent/avirulent pathotype combinations, and suggests that prior exposure of a host to low concentrations of virulent inoculum may compromise resistance and make plants more susceptible to subsequent exposure to high concentrations of avirulent inoculum.

Two of the three virulent pathotypes (2* and 5*) caused moderate clubroot symptoms at low concentrations. However, the third virulent pathotype (3*) did not cause significant levels of disease when it was applied only at low concentrations. The virulent pathotypes were represented by field isolates, which may consist of mixtures of different (avirulent and/or virulent) pathotypes in various proportions. It is likely, therefore, that for the field isolate classified as pathotype 3*, the number of virulent resting spores in the mixture was not sufficient to cause significant clubroot symptoms on CR canola at low concentrations. Higher concentrations were needed to increase the spore density of the virulent component of this field isolate to a level sufficient to cause disease. Previous studies with virulent isolates have found a positive correlation between clubroot severity and inoculum density (Hwang et al. 2017), and as expected, high inoculum concentrations of all three virulent pathotypes (PC1 and PC2) caused severe clubroot on the host cultivars. Notwithstanding the results with pathotype 3*, it appears that low or high concentrations of virulent isolates may cause clubroot and pose a significant threat to the production of canola. This is consistent with the findings of Strelkov et al. (2016; 2018) about elevated clubroot caused by the newly discovered *P. brassicae* isolates in Canada.

The most important finding of this study was the increased clubroot severity associated with previous exposure to low concentrations of virulent isolates, which may have compromised

host immunity or induced susceptibility in the host to avirulent isolates. Based on the disease rating data (Tables 2.1 and 2.2), clubroot severity and disease incidence were significantly higher in the ET compared with NC1 and NC2 for all pathotype combinations examined. For example, the mean DSI and DI values of seedlings inoculated with only a low concentration of pathotype 3* were elevated when the seedlings were inoculated with low concentrations of pathotype 3* followed by inoculation with high concentration of pathotype 3. This is similar to the induced susceptibility reported in barley and pea where preliminary inoculation with a compatible pathogen induces susceptibility to an incompatible pathogen or non-pathogen (Ouchi et al. 1974; Ouchi and Oku 1982; Ouchi 1983; Kunoh et al. 1985; Akpa and Archer 1994). Therefore, the phenotypes resulting from these interactions may reflect the virulence of pathotypes, specific combinations of isolates, host genotypes as well as environmental and other factors. Low concentrations of spores of virulent isolates causing moderate disease may also be explained by quorum sensing, which is a biological mechanism for microbial communication based on cell density. Quorum sensing has been reported to regulate the expression of virulence genes, which is a crucial step in pathogenesis (Albuquerque and Casadevall 2012). The presence of low concentrations of virulent spores may therefore inhibit the expression of virulence factors and multiplication of cells of the pathogen.

Relative quantification of *P. brassicae* DNA in relation to canola DNA in the 4455 collected root samples was achieved with qPCR assays. The comparative Ct qPCR method offers a high throughput assay for biomass quantification and has been used for the determination of pathogen biomass accumulation in many crops (Gachon and Saindrenan 2004; Gao et al. 2004; Marzachí and Bosco 2005; Feng et al. 2010; Weßling and Panstruga 2012). In contrast, microscopic examination requires cross-sectioning and staining of *P. brassicae*-infected roots as

well as hours of observation and counting of spores under the microscope (McDonald et al. 2014), which can make it unsuitable for determining pathogen biomass in such a large number of infected root samples.

In a study of different pathogenic species and plant hosts, Gachon and Saindrenan (2004) reported that *Alternaria brassicola* did not show significant variation in fungal DNA over the time-course of infection, while growth of *Botrytis cinerea* was rapid and increased steadily 72 h after inoculation of *Arabidopsis thaliana*. In a qPCR assay with the same primers used in this study and a TaqMan probe (TCPb1), Cao et al. (2014) observed that *P. brassicae* DNA increased significantly in susceptible and moderately resistant *Brassica* hosts from 5 to 42 dai. In contrast, *P. brassicae* DNA concentration in the highly resistant host ECD 02 increased from 5 to 20 dai and then decreased to levels lower than the concentration of *P. brassicae* DNA in the susceptible or moderately resistant hosts. The results of our comparative Ct qPCR assay suggested that the RQs of *P. brassicae* biomass to canola of root samples taken 1 to 17 dai were in most cases not significantly different from each other in all nine pathotype-cultivar treatment combinations.

The comparatively longer time taken for *P. brassicae* biomass to be detected in the present study may be in part due to differences in the genetics of the hosts used in the different studies. The three canola cultivars ‘‘45H29’’, ‘L135C’ and ‘L241C’ used in this study are resistant to pathotypes 2, 3 and 5. In contrast, the canola cultivar ‘‘Westar’’ used by Feng et al. (2013) is a universally clubroot-susceptible host, while the cultivar ‘Zephyr’ used by McDonald et al. (2014) is resistant to pathotype 6 (mainly occurring in cruciferous vegetables in Canada), but highly susceptible to pathotype 3. In the ET, PC1 and PC2, the relative pathogen to canola biomass ratio increased steadily from 21 to 35 dai, but remained mainly at non-significant levels in NC1 and NC2 throughout the time-course of the experiment. It was not until 28 to 35 dai

when there was sufficient *P. brassicae* biomass to be detected reliably by the comparative Ct qPCR method (Figure 2.5). Sundelin et al. (2011) also identified 35 dai as the optimal time-point at which *P. brassicae* was metabolically active both in the club and non-clubbed parts of the root, which is consistent with the findings of the current study.

In conclusion, this study found that inoculation with low concentrations of virulent *P. brassicae* isolates could prime or facilitate subsequent infection of the host by avirulent isolates. Additional studies are needed, however, to examine the impact of such inoculations on the defense response by the host. Moreover, the function of virulence genes during primary infection and the role of effectors in host defense are still unresolved and need to be investigated for a better understanding of clubroot pathogenesis.

Table 2.1. The effect of low concentrations of virulent *Plasmodiophora brassicae* pathotypes on disease severity (DSI %) on clubroot resistant canola cultivars

Pathotype[w]	Treatment[x]	Inoculation method	Mean DSI ± standard error (range) on canola cultivar[y,z]		
			"45H29"	'L135C'	'L241C'
2*/2	ET	10^3 spores/mL of pathotype 2* + two days later 10^7 spores/mL of pathotype 2	**37.9 ± 8.4** [b] (22.0 - 50.3)	**32.8 ± 6.9** [b] (19.4 - 42.3)	**43.1 ± 15.0** [b] (14.2 - 64.4)
	NC1	10^7 spores/mL of pathotype 2	**10.2 ± 4.0** [c] (3.5 - 17.3)	**2.3 ± 1.7** [c] (0.0 - 5.7)	**1.9 ± 1.1** [c] (0.0 - 3.9)
	NC2	10^3 spores/mL of pathotype 2*	**21.9 ± 8.0** [c] (6.8 - 33.9)	**15.0 ± 4.8** [c] (7.2 - 23.9)	**39.3 ± 17.3** [b] (6.3 - 64.8)
	PC1	10^3 spores/mL of pathotype 2* + two days later 10^7 spores/mL of pathotype 2*	**91.9 ± 1.6** [a] (88.9 - 94.5)	**91.1 ± 3.1** [a] (84.9 - 94.7)	**89.7 ± 3.0** [a] (84.6 - 95.1)
	PC2	10^7 spores/mL of pathotype 2*	**100.0 ± 0.0** [a] (100.0-100.0)	**97.7 ± 1.9** [a] (93.8 - 100.0)	**99.7 ± 0.3** [a] (99.1 - 100.0)
3*/3	ET	10^3 spores/mL of pathotype 3* + two days later 10^7 spores/mL of pathotype 3	**25.9 ± 5.6** [q] (14.8 - 32.5)	**23.3 ± 8.7** [q] (13.6 - 40.6)	**27.0 ± 8.9** [q] (17.1 - 44.8)
	NC1	10^7 spores/mL of pathotype 3	**12.0 ± 3.8** [r] (4.3 - 16.3)	**4.0 ± 3.1** [r] (0.0 - 10.1)	**2.7 ± 2.1** [r] (0.0 - 6.9)
	NC2	10^3 spores/mL of pathotype 3*	**2.3 ± 0.3** [r] (1.8 - 2.8)	**2.0 ± 1.7** [r] (0.0 - 5.5)	**1.3 ± 0.9** [r] (0.0 - 3.0)
	PC1	10^3 spores/mL of pathotype 3* + two days later 10^7 spores/mL of pathotype 3*	**94.4 ± 2.6** [p] (89.4 - 97.7)	**88.0 ± 4.3** [p] (83.3 - 96.6)	**89.7 ± 5.0** [p] (79.8 - 95.9)
	PC2	10^7 spores/mL of pathotype 3*	**89.9 ± 2.8** [p] (85.5 - 95.1)	**90.5 ± 3.6** [p] (83.3 - 94.7)	**90.1 ± 4.5** [p] (83.3 - 98.7)
5*/5	ET	10^3 spores/mL of pathotype 5* + two days later 10^7 spores/mL of pathotype 5	**37.6 ± 3.7** [y] (30.3 - 41.4)	**34.8 ± 3.7** [y] (27.6 - 40.0)	**44.0 ± 13.7** [y] (16.7 - 58.3)
	NC1	10^7 spores/mL of pathotype 5	**6.9 ± 1.6** [z] (4.8 - 10.0)	**4.0 ± 3.1** [w] (0.0 - 10.1)	**3.0 ± 2.4** [z] (0.0 - 7.9)
	NC2	10^3 spores/mL of pathotype 5*	**20.1 ± 9.0** [z] (2.4 - 31.5)	**17.4 ± 3.2** [z] (11.6 - 22.6)	**30.3 ± 14.9** [yz] (0.8 - 48.5)
	PC1	10^3 spores/mL of pathotype 5* + two days later 10^7 spores/mL of pathotype 5*	**96.4 ± 0.2** [x] (95.9 - 96.7)	**91.2 ± 3.8** [x] (83.7 - 95.6)	**95.5 ± 1.0** [x] (94.4 - 97.6)
	PC2	10^7 spores/mL of pathotype 5*	**98.5 ± 1.5** [x] (95.5 - 100.0)	**95.2 ± 0.9** [x] (93.4 - 96.3)	**97.0 ± 2.3** [x] (92.4 - 99.6)

[w] Three *P. brassicae* pathotypes (2*, 3* and 5*) used in the inoculation experiments could overcome resistance in the three canola cultivars "45H29", 'L135C' and 'L241C' while the three pathotypes (2, 3 and 5) could not. [x] Treatment designation: ET, Experimental treatment, NC, Negative control (NC1 and NC2) and PC, Positive control (PC1 and PC2). [y] The mean disease severity index (DSI %) ± standard error for three repetitions is indicated in boldface while the range of DSI for the three repeated experiments is in brackets and in regular font. For each genotype × pathogen treatment, the same superscripts above the "Mean DSI ± standard error" values indicate no significant difference ($P < 0.05$) between treatments, while different letters indicate significant differences in treatments. [z] Analysis of significance was conducted within pathotype pairs. DSI values in all experimental treatments (ET) were significantly higher than in NC1; the DSI values in seven ET were significantly higher than in NC2, whereas in two others (2*/2 and 5*/5 × 'L241C') there was no significant difference. All PC1 and PC2 were significantly higher than ET,

NC1 and NC2.

Table 2.2. The effect of low concentrations of virulent *Plasmodiophora brassicae* pathotypes on disease incidence (DI %) on clubroot resistant canola cultivars

Pathotype[w]	Treatment[x]	Inoculation method	Mean DI ± standard error (range) on canola cultivar[y,z]		
			"45H29"	'L135C'	'L241C'
2*/2	ET	10^3 spores/mL of pathotype 2* + two days later 10^7 spores/mL of pathotype 2	**58.8 ± 14.0** [b] (30.9 - 75.4)	**57.9 ± 8.7** [b] (43.0 - 73.1)	**65.4 ± 20.3** [ab] (24.7 - 86.1)
	NC1	10^7 spores/mL of pathotype 2	**17.9 ± 7.2** [c] (8.1 - 32.0)	**7.0 ± 5.2** [d] (0.0 - 17.1)	**5.8 ± 3.4** [c] (0.0 - 11.8)
	NC2	10^3 spores/mL of pathotype 2*	**38.2 ± 11.2** [bc] (18.1 - 56.7)	**30.0 ± 5.6** [c] (20.3 - 39.6)	**58.2 ± 21.1** [b] (16.7 - 85.9)
	PC1	10^3 spores/mL of pathotype 2* + two days later 10^7 spores/mL of pathotype 2*	**100.0 ± 0.0** [a] (100.0 - 100.0)	**99.6 ± 0.4** [a] (98.7 - 100.0)	**97.1 ± 2.9** [ab] (91.3 - 100.0)
	PC2	10^7 spores/mL of pathotype 2*	**100.0 ± 0.0** [a] (100.0 - 100.0)	**99.0 ± 1.0** [a] (97.1 - 100.0)	**100.0 ± 0.0** [a] (100.0 - 100.0)
3*/3	ET	10^3 spores/mL of pathotype 3* + two days later 10^7 spores/mL of pathotype 3	**46.9 ± 12.4** [q] (22.2 - 60.3)	**40.3 ± 17.3** [q] (22.4 - 75.0)	**44.2 ± 13.3** [q] (26.7 - 70.2)
	NC1	10^7 spores/mL of pathotype 3	**15.0 ± 4.8** [r] (5.9 - 22.2)	**11.9 ± 9.4** [r] (0.0 - 30.4)	**7.6 ± 6.6** [r] (0.0 - 20.7)
	NC2	10^3 spores/mL of pathotype 3*	**3.4 ± 1.0** [r] (1.8 - 5.1)	**2.9 ± 2.0** [r] (0.0 - 6.8)	**2.5 ± 1.5** [r] (0.0 - 5.2)
	PC1	10^3 spores/mL of pathotype 3* + two days later 10^7 spores/mL of pathotype 3*	**98.6 ± 0.8** [p] (97.2 - 100.0)	**98.8 ± 0.6** [p] (98.1 - 100.0)	**99.1 ± 0.9** [p] (97.4 - 100.0)
	PC2	10^7 spores/mL of pathotype 3*	**95.2 ± 2.4** [p] (92.3 - 100.0)	**96.5 ± 2.2** [p] (92.3 - 100.0)	**94.2 ± 2.3** [p] (90.8 - 98.7)
5*/5	ET	10^3 spores/mL of pathotype 5* + two days later 10^7 spores/mL of pathotype 5	**56.5 ± 5.9** [y] (44.8 - 62.9)	**52.8 ± 3.6** [y] (48.4 - 60.0)	**55.6 ± 12.7** [y] (30.3 - 70.0)
	NC1	10^7 spores/mL of pathotype 5	**7.9 ± 2.1** [w] (5.5 - 12.0)	**11.1 ± 8.2** [z] (0.0 - 27.0)	**8.4 ± 6.6** [z] (0.0 - 21.3)
	NC2	10^3 spores/mL of pathotype 5*	**31.4 ± 14.1** [z] (3.7 - 50.0)	**24.9 ± 5.5** [z] (14.1 - 32.1)	**40.0 ± 19.3** [yz] (2.3 - 65.9)
	PC1	10^3 spores/mL of pathotype 5* + two days later 10^7 spores/mL of pathotype 5*	**99.6 ± 0.4** [x] (98.9 - 100.0)	**100.0 ± 0.0** [x] (100.0 - 100.0)	**100.0 ± 0.0** [x] (100.0 - 100.0)
	PC2	10^7 spores/mL of pathotype 5*	**99.0 ± 1.0** [x] (97.0 - 100.0)	**100.0 ± 0.0** [x] (100.0 - 100.0)	**99.0 ± 1.0** [x] (97.1 - 100.0)

[w] Three *P. brassicae* pathotypes (2*, 3* and 5*) used in the inoculation experiments could overcome resistance in the three canola cultivars "45H29", 'L135C' and 'L241C' while the three pathotypes (2, 3 and 5) could not. [x] Treatment designation: ET, Experimental treatment, NC, Negative control (NC1 and NC2) and PC, Positive control (PC1 and PC2). [y] The mean disease incidence (DI %) ± standard error for three repetitions is indicated in boldface while the range of DI for the three repeated experiments is in brackets and in regular font. For each genotype × pathogen treatment, the same superscripts above the "Mean DI ± standard error" values indicate no significant difference ($P < 0.05$) between treatments, while different letters indicate significant differences in treatments. [z] Analysis of significance was conducted within pathotype pairs.
DI values in all ET were significantly higher than in NC1; the DI values in six ET were significantly higher than in NC2, whereas three others

(2*/2× ‘45H29’, 2*/2 and 5*/5 × ‘L241C’) showed no significant difference. All PC1 and PC2 were significantly higher than ET, NC1 and NC2.

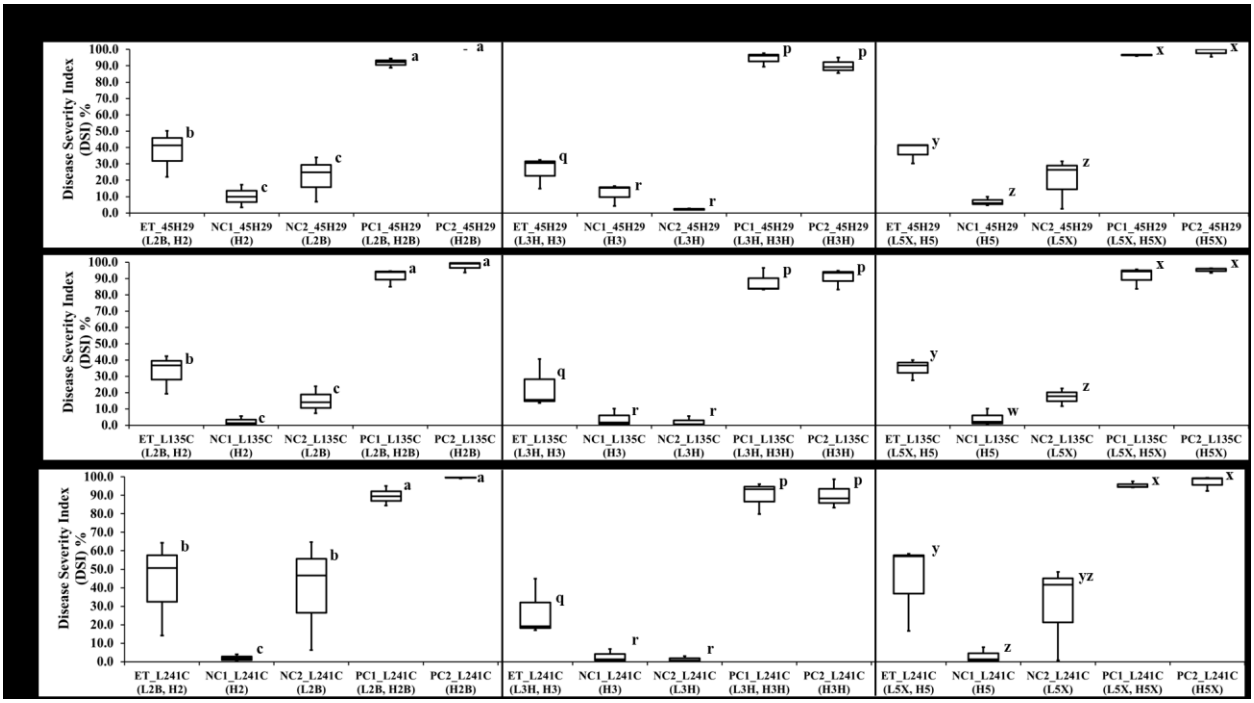

Figure 2.1. Disease severity index in all nine pathotype-cultivar combinations. For each genotype × pathogen treatment, the same superscripts above the whisker plot values indicate no significant ($P < 0.05$) difference between treatments while different letters indicate significant differences in the treatments.

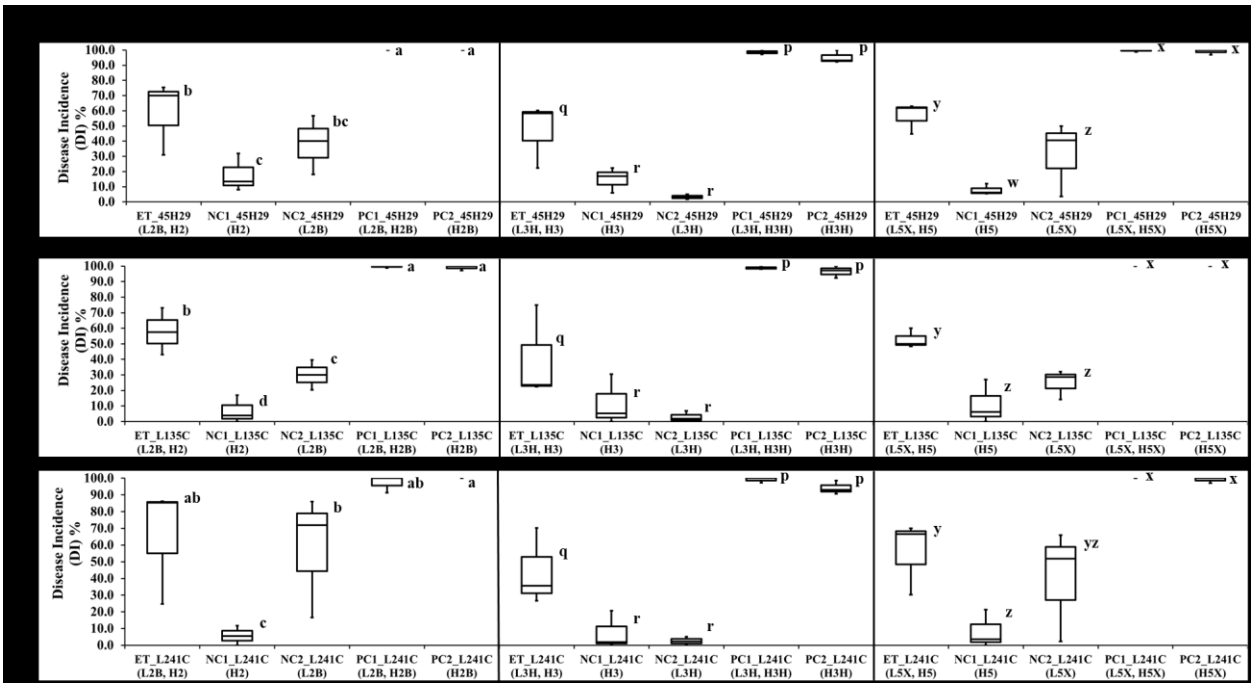

Figure 2.2. Disease incidence in all nine pathotype-cultivar combinations. For each canola genotype × pathogen treatment, the same superscripts above the whisker plot values indicate no significant ($P < 0.05$) difference between treatments while different letters indicate significant differences in the treatments.

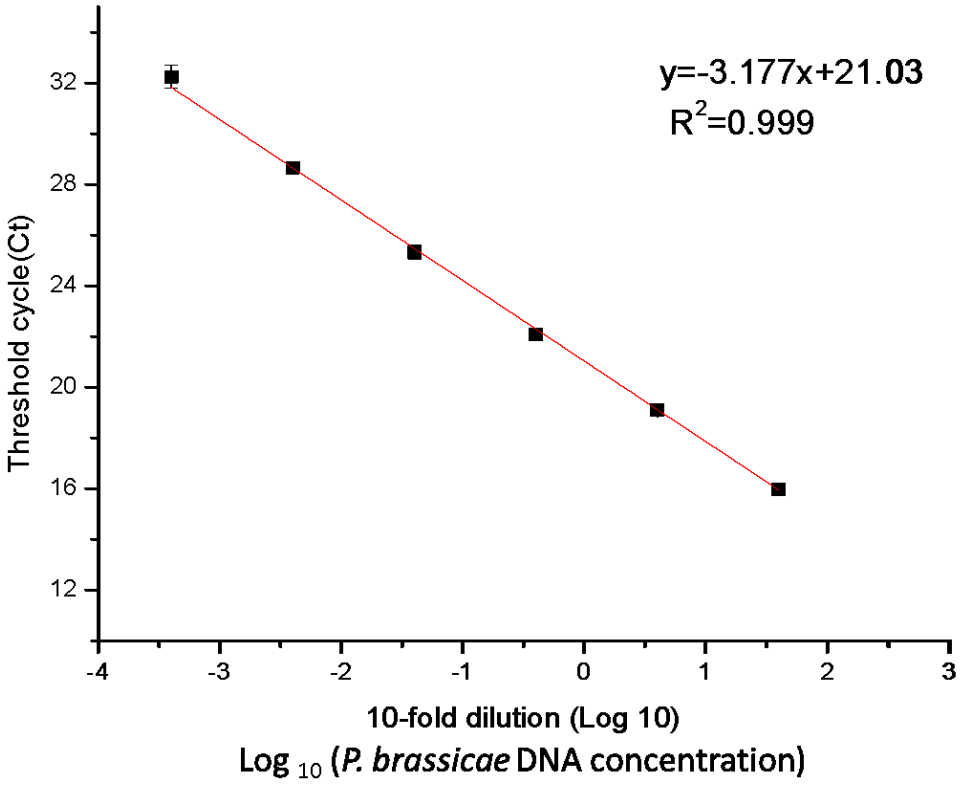

Figure 2.3. Calibration curve: Ct vs. log (*Plasmodiophora brassicae* DNA). Standard curve created by plotting threshold cycle (Ct) against a 10-time dilution series of *P. brassicae* genomic DNA starting from 40ng/µL. This experiment was conducted and repeated by using *P.brassicae* DNA from an independent assay.

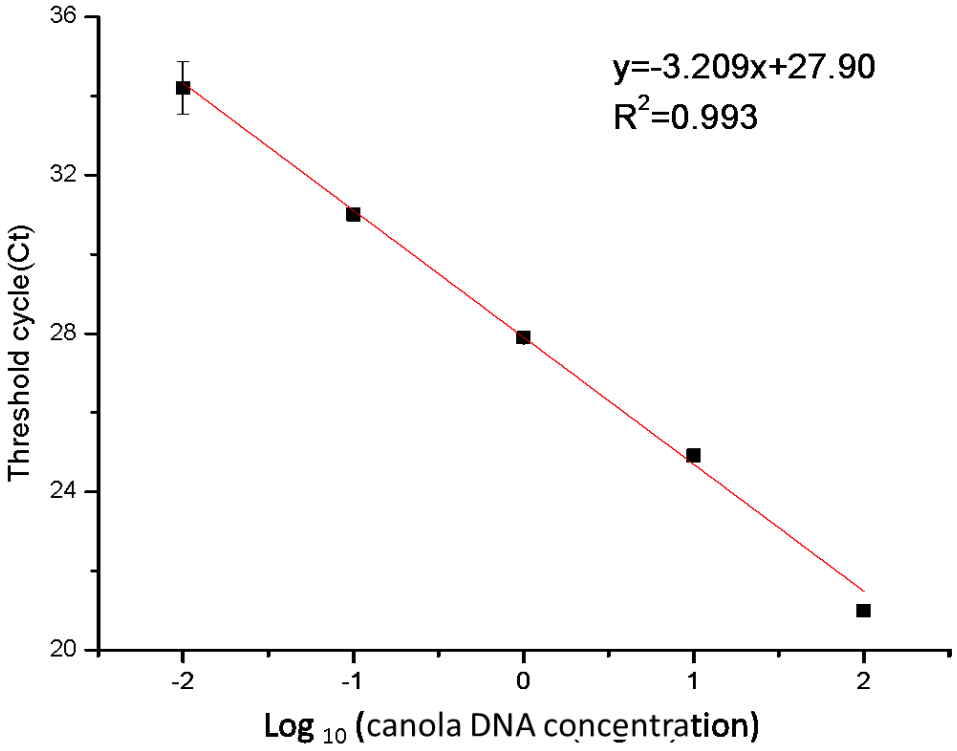

Figure 2.4. Calibration curve: Ct vs. log (canola DNA). Standard curve created by plotting threshold cycle (Ct) against a 10-time dilution series of canola genomic DNA starting from 100ng/µL. This experiment was conducted and repeated by using canola DNA from an independent assay.

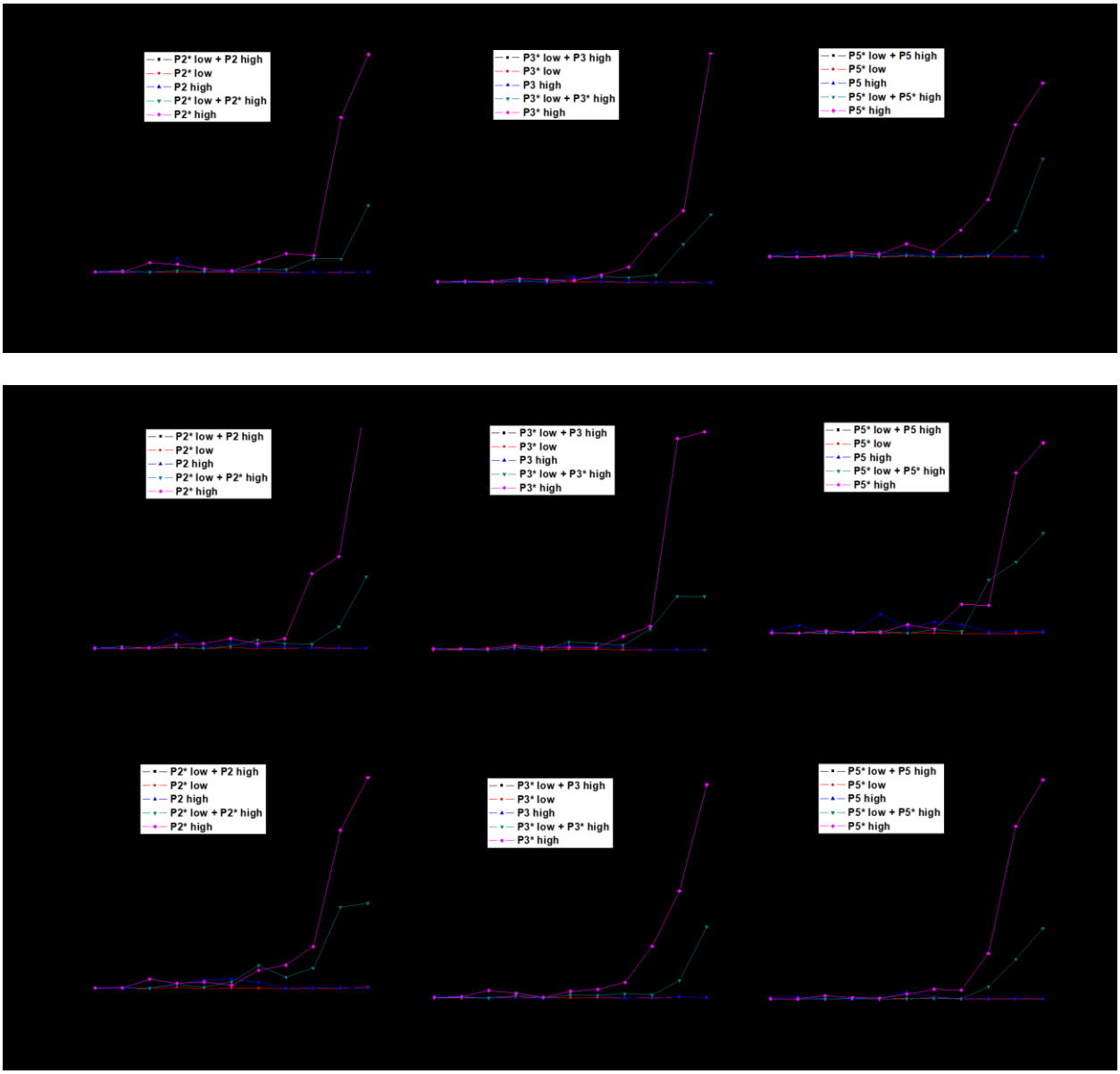

Figure 2.5. Clubroot disease progression in the canola cultivars ‘‘45H29’’ (a), ‘L135C’ (b) and ‘L241C’(c). Results were normalized by the ΔΔ cycle threshold method with β-actin gene as the reference gene. All experiments were repeated three times. The Y-axis represents the *P. brassicae* DNA amount relative to plant DNA in the root.

Chapter 3: Differential Gene Expression by Two Pathotypes of *Plasmodiophora brassicae* During Infection of Canola (*Brassica napus*)

3.1 Introduction

Clubroot, caused by the obligate parasite *Plasmodiophora brassicae* Woronin, is an important disease of Brassica crops, causing the formation of large galls or clubs on the roots of susceptible hosts. In Alberta, clubroot is a major threat to canola (*Brassica napus* L.) production (Hwang et al. 2012) and causes yield losses in excess of 30% in severely infested fields (Tewari et al. 2005). The number of fields with confirmed *P. brassicae* infestations has increased sharply in this province, from just 12 in 2003 (Strelkov et al. 2005; Tewari et al. 2005) to more than 3,000 by 2018 (Strelkov et al. 2019). A characteristic of biotrophic pathogens is the establishment of a long term parasitic relationship with their host, without killing the plant cells or tissues (Pietro 2012), while at the same time avoiding activation of the host defense responses (Vleeshouwers and Oliver 2014). Studies of the mechanisms of pathogenicity in *P. brassicae* have been limited, yet are warranted, given the economic significance of this pathogen.

The life cycle of *P. brassicae* includes three stages: (1) survival in the soil as dormant resting spores, (2) germination of resting spores and root hair infection by primary zoospores, and (3) secondary infection of the host by secondary zoospores (Ayers 1944; Ingram and Tommerup 1972). Primary infection is initiated by attachment and penetration of the root hairs by primary zoospores. After several cycles of cleavage, 4-16 secondary zoospores are produced (Feng et al. 2012a). Primary infections do not result in the development of macroscopic symptoms, with the secondary infections causing the root malformations and possibly playing a more important role in pathogenesis (Kageyama and Asano 2009). Primary infection occurs not only in host plants, but also in non-host plants such as *Lolium perenne*, *Reseda odorata*, and

Tropaolum majus (MacFarlane 1952; Feng et al. 2012b). Secondary zoospores cause the secondary infection of the host root cortical tissue. However, the mechanisms used by secondary zoospores to cause cortical infection are not fully understood (Kageyama and Asano 2009). Ludwig-Müller et al. (1999) reported that *P. brassicae* might also cause secondary infection in non-host plants. Secondary zoospores give rise to secondary plasmodia, which eventually give rise to large numbers of resting spores. Hypertrophy and hyperplasia of the host root tissues, which result in the galls typical of clubroot, occur during the secondary infection stage. As the galled roots decompose, the resting spores are released into the soil, serving as inoculum for future infections. Morphologically, primary zoospores and secondary zoospores are difficult to distinguish (Kageyama and Asano 2009), but differences in their ability to cause root malformation indicates that these differences are modulated by physiological processes.

Many approaches have been proposed for the control of clubroot (Hwang et al. 2014; Peng et al. 2014), with the planting of resistant cultivars usually regarded as most effective (Rahman et al. 2014). Several clubroot resistant canola hybrids have been introduced to the Canadian market (Strelkov and Hwang 2014; Peng et al. 2014), but the resistance in these cultivars has been overcome in an increasing number of fields in recent years (Strelkov et al. 2016; Strelkov et al. 2018). Understanding of clubroot pathogenesis, especially at the gene expression level, is limited (Hwang et al. 2012), but may contribute to knowledge-based and novel strategies to develop clubroot resistant canola. The *Brassica rapa* spp. *chinensis* clubroot resistance gene *Rcr1* was mapped on the linkage group A03 and its functional annotation indicates it may be involved in the inhibition of clubroot symptom development (Chu et al. 2014). A more recent study of the molecular mechanisms of *Rcr1*-mediated resistance indicated that plants expressing this gene showed significantly increased levels of lignin, phenolic

compounds and other components of the cell wall (Lahlali et al. 2017). On the pathogen side of things, Bulman et al. (2006) screened 232 clones derived from a suppression subtractive hybridization (SSH) library and identified 76 *P. brassicae* genes potentially related to pathogenicity. Feng et al. (2013) used all of the 118 *P. brassicae* genes available at that time and assessed their expression in primary and secondary zoospores, finding that 58 genes were up-regulated and 55 were down-regulated in the secondary zoospores. A few reports suggested that some genes are present in both the *P. brassicae* (Bulman et al. 2006) and *B. rapa* (Lee et al. 2008) genomes.

Most of the studies noted above were conducted before the release of the whole *P. brassicae* genome, which was estimated to be 25.5Mb in size with a total of 9370 predicted genes (Schwelm et al. 2015). The availability of the *P. brassicae* genome sequence, along with the development of molecular techniques such as quantitative PCR (qPCR), enables more in-depth exploration of clubroot pathogenesis than was previously possible. The delta-Ct (ΔCt) qPCR method, developed to quantify target gene expression level (Winer et al. 1999), has been used in many studies of pathogen gene expression *in planta* (Gao et al. 2004; Gachon and Saindrenan 2004; Marzachí and Bosco 2005; Feng et al. 2010; Weßling and Panstruga 2012). The aim of the current study was to identify genes that are differentially expressed during infection of canola by two pathotypes of *P. brassicae*, based on the hypothesis that the differentially expressed genes might contribute to the avirulent and virulent phenotypes observed.

3.2 Materials and methods

3.2.1 Plant materials

Two *B. napus* cultivars, '45H29' and 'Westar', were used to study genes that are differentially expressed during clubroot pathogenesis. The former ('45H29') is a hybrid cultivar with a broad-spectrum resistance to pathotypes 2, 3, 5, 6 and 8 of *P. brassicae*, while the latter ('Westar') is an open-pollinated cultivar susceptible to all of these pathotypes.

3.2.2 Pathogen isolations and inoculum preparation

One single-spore isolate (ORCA-ss3) and one field isolate (L-G1) of *P. brassicae*, classified as pathotype 5 on the system of Williams (1966), were included in this study (Xue et al. 2008; Strelkov et al. 2016). While both isolates shared the same virulence pattern on the differentials of Williams, the field isolate L-G1, unlike ORCA-ss3, is able to overcome the resistance in most clubroot resistant canola cultivars including '45H29' (Strelkov et al. 2016), and is commonly referred to as pathotype 5X (Strelkov et al. 2018). Both isolates were maintained as galls on the roots of the Chinese cabbage (*B. rapa* L.) 'Granaat'. To prepare inoculum, the clubbed roots were ground in sterile distilled water (sdH_2O) with a mortar and pestle and filtered through eight layers of cheesecloth. The resting spore concentration was measured with a hemocytometer as described by Strelkov et al. (2006), and adjusted to 2.3×10^7 spores mL^{-1} with sdH_2O for use as inoculum. Resting spore suspensions were stored at 4°C and used within 24 h after preparation.

3.2.3 Inoculation, sample collection and disease assessment

Host reactions to *P. brassicae* pathotypes 5 (P5I) and 5X (P5X) were assessed in greenhouse bioassays. Briefly, the rootlets of 7-day old seedlings of '45H29' and 'Westar', which had been germinated on moistened sterilized filtered paper in Petri dishes, were dipped in a resting spore suspension for 10 sec (Strelkov et al. 2006). The inoculated seedlings were then transplanted into cups (7 cm × 12 cm) filled with Sunshine Mix 4 potting mixture (Sun Gro Horticulture Canada Ltd., Seba Beach, AB, Canada), at a density of five seedlings per pot. Holes were made at the

bottom of the cups to allow for drainage, and they were moved to a greenhouse supplemented with artificial lighting to maintain a 16 h day/8 h day/night schedule at 20-25°C and 15-18°C, respectively. The cups were placed on trays filled with slightly acidified water (pH = 6.0) to create conditions favorable for disease development. Twenty days after inoculation, the cups were removed from the water-filled trays, watered daily and fertilized (N: P: K=1: 2: 1) twice a week.

The experimental set-up for '45H29' consisted of three replicated experiments for the biomass experiments and four replicated experiments for the gene expression studies, each with P5I and P5X. Each biological replicate consisted of 3 cups (15 seedlings) in the biomass experiments and 4 cups (20 seedlings) in the gene expression studies. Thus, 45 and 80 seedlings of '45H29' were assessed for each of pathotypes P5I and P5X, respectively, in the biomass and gene expression studies. The remaining 9 cups (45 seedlings per cultivar) were assessed for clubroot disease severity after 7 weeks. Treatments were arranged in a completely randomized design. The same assessments were conducted with 'Westar' following inoculation with P5I, except that the seedlings were sampled only for gene expression and clubroot severity and not biomass. Forty-five 'Westar' seedlings were also inoculated with P5X in three biological replicates to serve as positive controls for disease severity.

Root samples were collected at 7, 14 and 21 days after inoculation (dai). Briefly, the aboveground material was cut-off at the crown and the roots were washed under tap water to remove potting mix and other debris. The root samples were stored at -80°C for analysis of gene expression and at -20°C for measurements of biomass. The remaining plants from each experiment were assessed for clubroot disease development 7 weeks after inoculation. Disease assessment was carried out on the 0-3 rating scale of Kuginuki et al. (1999), where: 0 = no galls;

1 = a few small or bead-sized galls on < 1/3 of the roots; 2 = medium galls on 1/3-2/3 of the roots, and 3 = large galls on > 2/3 of the roots. An index of disease severity (DSI) was calculated based on the formula of Horiuchi and Hori (1980) as modified by Strelkov et al. (2006):

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

Where: n is the number of plants in each class; N is the total number of plants; and 0, 1, 2 and 3 are the symptom severity classes.

3.2.4 DNA and RNA extraction

For DNA extraction, five roots collected from one biological replicate at each of 7, 14, and 21 dai were pooled, flash-frozen in liquid nitrogen and homogenized in a mortar with a pestle. Replicates were kept separate. Total genomic DNA was extracted using the cetyltrimethyl ammonium bromide (CTAB) method (Sambrook and Russell 2001). For RNA extraction, six roots collected from one biological replicate at each of 7, 14, and 21 dai were pooled, flash-frozen and homogenized as above, with total RNA extracted using a PureYield RNA Midiprep System Kit (Promega, Madison, WI) following the manufacturer's protocol.

3.2.5 Selection of database sequences and candidate genes

A total of 9784 putative *P. brassicae* protein sequences (08/2015) were retrieved from the GenBank database of the National Center for Biological Information (www.ncbi.nlm.nih.gov), which included, but was not limited to the entries from the whole genome sequencing study (Schwelm et al. 2015). Criteria for the selection of the putative protein sequences were the presence of an N-terminal signal peptide as predicted by SignalP software (Bendtsen et al. 2004), a high cysteine content (>2.9%), and an N-terminal conserved RXLR motif. Candidate genes meeting these criteria were aligned with BioEdit Sequence Alignment Editor (Hall 2011) to

remove redundancies. The predicted functions of proteins encoded by genes with unique sequences were determined using the BLASTp and tBLASTn algorithms, with a cut-off E-value of 0.0001 (Calvet et al. 2016) considered significant.

3.2.6 Primer design and validation

Forward and reverse primers were designed using Primer 3 (Untergasser et al. 2012) from the exon regions of genes coding for proteins with predicted functions. Desirable primer pairs were 18-23 bp in length, which produced amplicons that ranged from 100-250 bp but also had a melting temperature of 57-63°C and GC% values of 30-70%. All target primers developed in this study were pre-screened by qPCR using cDNA (see below) from two biological replicates of the P5X/'45H29' and P5I/'45H29' treatments at 14 dai. All results were normalized relative to the *P. brassicae* gene Pbr018 (Feng et al. 2013). Primers showing potential differential expression at 14 dai were used in qPCR analysis of cDNA from all four biological replicates of the P5X/'45H29', P5I/'45H29' and P5I/'Westar' treatments at 7, 14 and 21 dai, as described below. Each biological replicate consisted of two technical repeats.

3.2.7 Quantitative PCR and cycling conditions

To compare the expression levels of the selected genes and to determine the relative amounts of *P. brassicae* in the roots of '45H29' following inoculation with P5I and P5X, a SYBR Green-based qPCR assay was used. One primer set (F: GGGACATCACCGACTACCTG; R: ACTGCTCCGAGTTGGACATC) designed based on the *P. brassicae* reference gene (PRG) *Pbr018* (Feng et al. 2013) and another (F: ACGAGCTACCTGACGGACAAG; R: GAGCGACGGCTGGAAGAGTA) based on the canola reference gene (CRG) *BnACT2* (Yang et al. 2009) were used for the relative quantification of *P. brassicae* DNA and normalization, respectively. Normalization was necessary to minimize yield differences during DNA extraction.

Quantification of the pathogen to plant biomass ratio in the infected roots was calculated according to the comparative threshold cycle (Ct) by the delta Ct method (pathogen/plant genomic DNA fold change = $2^{-\Delta Ct} = 2^{-(Ct\,(PRG) - Ct\,(CRG))}$).

Reverse transcription was performed with a GoTap® 2-Step RT-qPCR System kit (Promega), while q-RT-PCR analyses were conducted in a StepOne Real-Time PCR System (Fisher Canada, Ottawa, ON). The reaction mixtures for q-RT-PCR analysis included GoTaq qPCR Master Mix, cDNA template, and the target and reference gene primers. Cycling conditions consisted of one cycle at 95°C for 10 min pre-denaturation, 40 cycles of a two-step procedure (15 sec at 95°C for denaturation, 1 min at 60°C for annealing and extension), and a final step from 60 to 95°C. A standard curve was generated from 80 ng of *P. brassicae* DNA serially diluted to 0.008ng.

3.2.8 Statistical analyses

Data obtained from the gene expression experiments were analyzed with the statistics software R (R Development Core Team 2015) using the "Nest Design" (Hierarchical Design) model at a significance level of $P < 0.01$. The linear model used to analyze a hierarchical design was $y_{ijk} = \mu + A_i + B_{(i)j} + \varepsilon_{k(ij)}$, where: i indexed the pathotypes, j indexed the biological replicates within each pathotype, and k indexed the technical repeats nested within biological replicates. The "Lme" package was used to calculate pathotype effects on gene expression level during pathogenesis. Transcripts with a ≥ 2-fold difference in abundance were also regarded as potentially differentially expressed (Zuluaga et al. 2016).

3.3 Results

3.3.1 Clubroot symptom developments

No visible symptoms of clubroot were observed on '45H29' at 7 dai with either pathotype 5I or 5X. At 14 dai, some swelling of the roots was visible in response to inoculation with P5X, but plants inoculated with P5I remained symptom-free. At 21 dai, more severe galling began to develop on '45H29' inoculated with P5X, while the plants inoculated with P5I still appeared healthy to the naked eye. The final assessment at 7-weeks revealed severe clubroot (DSI = 99% ± 0.01) on '45H29' in response to P5X, but only very mild symptoms (DSI = 10% ± 0.00) in plants inoculated with P5I (Table 3.1, Figure 3.1). These results confirmed that '45H29' is susceptible to P5X and resistant to P5I. As expected 'Westar', the susceptible control, developed severe clubroot in response to inoculation with both pathotypes.

3.3.2 Primer development and validation

Of the 9784 *P. brassicae* putative protein sequences downloaded from the NCBI, 1,094 contained a signal peptide, of which 205 sequences were unique. These included 160 genes encoding proteins with a high (>2.9%) cysteine content, and 45 genes encoding proteins carrying an N-terminal conserved RXLR motif. Thus, a total of 205 forward and reverse primer pairs were designed and used in the gene expression analyses.

3.3.3 DNA standard curve for *P. brassicae*

The standard curve generated with *P. brassicae* DNA showed a strong negative and linear relationship between threshold cycle (Ct) and *P. brassicae* DNA concentration (Figure 3.2). The high R^2 value (0.999) indicated favorable reaction conditions. Quantitative PCR with the primers designed based on the *P. brassicae* reference gene *Pbr018* did not amplify any product from genomic DNA extracted from healthy (non-inoculated) roots of '45H29' and 'Westar', confirming the specificity of this primer set for the pathogen.

3.3.4 Pathogen biomass quantification

The biomass of P5X relative to '45H29' (P5X/'45H29') increased by ca. 130% (0.07 to 0.16) from 7 to 14 dai, while the increase from 14 to 21 dai was about 320,000% (0.16 to 51.94) (Figure 3.3). In contrast, the biomass of P5I relative to '45H29' (P5I/'45H29') decreased by about 90% (1.52 to 0.17) from 7 dai to 14 dai, and decreased further by about 30% (0.17 to 0.12) from 14 to 21 dai (Figure 3.3).

3.3.5 Pathogen gene expression profiles in '45H29'

After RNA extraction and reverse transcription, all 205 target genes were pre-screened for differential expression at 14 dai by qPCR. Analysis of the gene expression data indicated that 140 genes produced no product and 49 genes showed no difference in transcript abundance in the roots of '45H29' inoculated with P5I or P5X. Transcript levels were higher for 15 of the remaining 16 genes in the root samples inoculated with P5I, while only one gene transcript was more abundant in samples inoculated with P5X (Table 3.3, Figures 3.4a and 3.4b). These 16 genes were selected for further expression analysis over a a longer time-course (7 and 21 dai). Analysis of the entire time-course indicated that none of the 16 genes produced amplifiable levels of product at 7 dai, while significant differences ($P \leq 0.01$) were observed in the transcript abundance of 11 of the 16 genes at 21 dai (Table 3.3, Figures 3.4a and 3.4b).

At 21 dai, nine genes (#1, 3, 4, 7, 8, 9,10, 11, 12) were more highly expressed following infection by P5X, while two genes (#6, 13) were more highly expressed following infection by P5I (Table 3.3, Figures 3.4a and 3.4b). From 14 dai to 21 dai, eight (#1, 3, 4, 5, 7, 9, 12, 15) of the 16 genes (Table 3.3, Figures 3.4a and 3.4b) showed contrasting expression patterns following inoculation with P5I versus P5X ($P \leq 0.01$). Expression of gene #14 was down-regulated during infection of '45H29' by both P5X and P5I, while genes #2 and 11 were upregulated in both cases. The expression of gene #6 did not show any significant changes during infection by P5X,

but was down-regulated during infection by P5I. In contrast, expression of the genes #8 and 16 was up-regulated following infection by P5X, but did not change after infection by P5I. Expression of two genes (#10 and 13) did not change from 14 to 21 dai for either pathotype (Table 3.3, Figures 3.4a and 3.4b). In the case of P5X, the transcript abundance of 12 of the 16 genes (#1, 2, 3, 4, 5, 7, 8, 9, 11, 12, 15, 16) increased throughout the time-course at 7, 14 and 21 dai. Only one gene (#14) showed up-regulation from 7 to 14 dai and down-regulation from 14 to 21 dai, while three genes (#6, 10 and 13) were up-regulated from 7 to 14 dai with expression remaining stable from 14 to 21 dai (Table 3.3, Figure 3.4a).

3.3.6 Transcript abundance in the P5I/'Westar' interaction

As was observed in the compatible P5X/'45H29' interaction, no product was amplified for any of the 16 genes at 7 dai in 'Westar' roots inoculated with P5I. The expression pattern of 12 of the genes (#1, 2, 3, 4, 5, 7, 8, 9, 11, 12, 15, 16) in the P5I/'Westar' interaction was similar to that in the P5X/'45H29' interaction, with most upregulated throughout the time-course (Table 3.3, Figure 3.4c). However, gene #6, which was down-regulated in P5X/'45H29', was up-regulated in P5I/'Westar' from 7 to 14 dai and down-regulated from 14 to 21 dai, and hence showed a different expression pattern than in the P5X/'45H29' interaction. The abundance of transcript of gene #10 increased from 7 to 21 dai in P5I/'Westar', while there was no significant difference in the expression of genes #13 and 14.

3.3.7 Identification of 16 differentially expressed genes

The best matches in the GenBank database for each of the 16 genes analyzed over the entire time-course are indicated in Table 3.4. No matches (E-value < 0.0001) were found for seven of the genes (#1, 3, 5, 6, 8, 10, and 11). Among those that had significant matches in GenBank, two genes (#9 and 15) matched secreted proteins, gene #4 matched an ATP-binding cassette, while

gene #7 matched a cell surface glycoprotein that also belongs to the ATP-binding cassette sub-family. Gene #16 matched a zinc finger protein, gene #2 shared 55% identity with the PbSUNK2 gene from *P. brassicae* (Bulman et al. 2006), and gene #13 matched a hypothetical protein BVRB 02880. The remaining genes matched an E3 ubiquitin-protein ligase (#13) and a pepsin II-like protein (#14).

3.4 Discussion

Pathogen biomass and gene expression were monitored at multiple time-points after inoculation of the *B. napus* genotypes '45H29' and 'Westar' with isolates representing pathotypes 5I and 5X of *P. brassicae* (Strelkov et al. 2016). Previous studies suggested that primary and secondary infection by *P. brassicae* occur at 0.5 – 12 dai and 3 – 6 dai, respectively (Asano et al. 2000; Kageyama and Asano 2009; Feng et al. 2012a), while club formation is initatied at 20 dai (Verma et al. 2014). Hence, in this study, samples were analyzed 7, 14 and 21 dai, with the final disease assessments made at 7 weeks after inoculation.

The observation that relative biomass of P5X increased while that of P5I decreased in '45H29' from 7 to 21 dai is consistent with the fact that these pathotypes are virulent and avirulent, respectively, on this host (Strelkov et al. 2016). The virulence types of both pathotypes on '45H29' and 'Westar' were confirmed in the disease assessments conducted at 7 weeks. Pathotype 5X is able to colonize and multiply in the host tissues, while growth of P5I is arrested or much more restricted. Nonetheless, at 7 dai, there was a higher relative pathogen biomass in the P5I/'45H29' interaction than in the P5X/'45H29' interaction. This may be explained, at least in part, by the fact that primary infection by *P. brassicae* can occur both in compatible and incompatible interactions (Ludwig-Müller et al. 1999; Feng et al. 2012b). The initiation of secondary infection, which later may have been inhibited by host resistance mechanisms, also

cannot be ruled out (McDonald et al. 2014). At the later stages of infection, at or beyond 14 dai, the relative biomass of P5I in '45H29' decreased, possibly as mature primary plasmodia released their secondary zoospores outside of the root via exit tubes (Fei et al. 2016).

Stergiopoulos and de Wit (2009) reported that the majority of *Avr* genes from filamentous microorganisms encode small, secreted proteins that are often cysteine-rich. Secreted cysteine-rich proteins constitute a common source of fungal effectors that can activate host resistance or susceptibility (Lu and Edwards 2016). The presence of an N-terminal signal peptide is also associated with secreted proteins (Blobel and Dobberstein 1975; Petre and Kamoun 2014), and an RXLR motif can define predicted effector repertories in many species (Jiang et al. 2008; Morgan and Kamoun 2007). Indeed, Whisson et al. (2007) found that an RXLR-EER motif serves as a translocation signal for delivery of *Phytophthora infestans* effectors into host cells. Given the importance of the RXLR motif, its presence was used a criterion in the selection of candidate genes to analyze in the current study.

The non-detection of quantifiable levels of transcript at 7 dai for the 16 genes analyzed over the entire time-course may have reflected several possibilities. Since primary infection occurs in both *P. brassicae* hosts and non-hosts (MacFarlane 1952; Feng et al. 2012b), there may have been no significant differences in gene expression in the compatible and incompatible interactions. Further, the genes may not be involved in pathogenicity during the early stages of the infection process, and perhaps have a role after primary infection.

At 14 dai, only one (#10) of the analyzed genes was expressed at a higher level in '45H29' during P5X vs. P5I infection, while 15 genes were expressed at higher levels in the same host during infection by P5I. A possible reason for most genes being expressed at lower

levels by P5X may be deduced from the observation that when biotrophic pathogens initiate infection, they try to secure a foothold and remain stealthy to avoid triggering host resistance (Vleeshouwers and Oliver 2014). Hence, perhaps expression of certain genes by the virulent P5X was modulated to avoid detection by the host.

At 21 dai, 11 of the 16 selected genes were expressed differentially during infection of '45H29' by P5I and P5X, with 9 genes more highly expressed by P5X and two by P5I. This suggests that by this time, P5X had already bypassed or overcome the so-called pathogen-associated molecular pattern (PAMP) triggering immunity (Jones and Dangl 2006), and had started to deploy effectors to increase virulence and (or) manipulate host metabolism. Twelve genes were up-regulated consistently throughout the time-course (7, 14 and 21 dai) following inoculation of 'Westar' with P5I and '45H29' with P5X infection. Since these were both compatible interactions, these genes may be important in pathogenesis or in modulating the host defense response from initial infection to macrosymptom development. The cloning and functional characterization of the 16 differentially expressed genes will help to elucidate their roles in clubroot pathogenesis. In the meantime, their similarity to entries in GenBank may provide some clues as to their function.

Seven genes (#1, 3, 5, 6, 8, 10 and 11) encoded proteins with no homologs in GenBank, and which could potentially be unique to *P. brassicae*. If this is confirmed with further study, these genes may be used for barcoding the pathogen. Gene #15 encoded a product that matched secreted proteins and also the enzyme polysaccharide deacetylase. This enzyme is involved in the deacetylation of chitin and xylan (Arnaouteli et al. 2015). Chitin is a very important chemical component (25.1%) in the cell wall of *P. brassicae* (Moxham and Buczacki 1983), while xylan is an important hemicellulous component in dicot cell walls (Varner and Lin 1989). Thus, this

enzyme may play a role during the infection process, helping to modify the resting spore and plant cell walls for successful penetration. It may also play a role in development of the secondary plasmodia whithin infected host cells. Gene #2 matched PbSUNK2, a high copy number gene first reported from *P. brassicae*-infected *Arabidopsis* tissues (Bulman et al. 2006). PbSUNK2 also was differentially expressed during infection of Chinese cabbage by *P. brassica*e (Sundelin et al. 2011), and is a good candidate to be a microbial effector since it is small (< 177 aa) and cysteine-rich (Stegiopoulus and de Wit 2009). Gene #16 encoded a product matching a zinc finger protein. Zinc finger proteins provide a structural motif to stabilize protein folding (Seim et al. 2013). Yue et al. (2015) reported that a putative Cys2-His2 (C2H2) zinc finger protein was essential for appressorium differentiation in *Magnaporthe oryzae*. Similarly, Cao et al. (2016) found that a C2H2 zinc finger protein is involved in fungal growth, appressorium formation, asexual development and pathogenicity. These reports suggest that gene #16 is important in pathogenesis. Genes #4 and 7 encoded proteins with mathching proteins belonging to the ATP binding cassette (ABC)-transporter sub-family; ABC-transporters have been found to help to energize cell biochemical reactions (Morita et al. 2015). A recent study indicated that the ABC-transporter CgABCF2 was required for appressorium formation and infection by *Colletotricum gloeosporioides* (Zhou et al. 2017). Finally, gene #13 shared similarity with an E3 ubiquitin-protein ligase; these proteins are reported to regulate the plant's innate immunity (Duplan and Rivas 2014).

Knowledge of the *B. napus*/*P. brassicae* interaction is important for our understanding and long-term management of clubroot. The differential proliferation of the pathogen in compatible and incompatible interactions, combined with changes in gene expression, highlight the many alterations occurring in infected tissues within the first 3 weeks of infection. Moreover,

the similar expression profiles for some of the genes examined in two different compatible interactions (P5X/‘45H29’ and P5I/‘Westar’) highlight these genes as of potential importance in establishing *P. brassicae* infection. Further study may confirm and clarify the role of the genes presented here, helping to elucidate their contribution to clubroot pathogenesis.

Table 3.1. Clubroot disease severity index (DSI) 7 weeks after inoculation of the *Brassica napus* genotypes '45H29' and 'Westar' with *Plasmodiophora brassicae* pathotypes 5I (P5I) and 5X (P5X).

Pathotype and cultivar	Sample size	DSI%	Range (DSI %)
P5I on '45H29'	(10, 10, 10)	10% ± 0.00*	10% -10%
P5X on '45H29'	(13, 12, 11)	99% ± 0.01	97% -100%
P5I on 'Westar'	(12, 12, 9)	100% ± 0.00	100% -100%
P5X on 'Westar'	(12, 11, 9)	100% ± 0.00	100% -100%

*Standard error

Table 3.2. Primer sequences designed to amplify 16 *Plasmodiophora brassicae* genes differentially expressed in *B. napus* '45H29' after inoculation with pathotypes 5I and pathotype 5X of the pathogen.

Gene #	Accession #	Forward primers	Reverse primers
1	CDSF01000057	CTGGACCTGCAAACAATCAG	CGAAGATGTTGTACGACCAG
2	CDSF01000079	AAAGTTGAGCCGTACATCAA	GACGACATAGCCATCATCAC
3	CDSF01000145	TAGAGCATGAACGCGAATAG	GTTGTCATCACCGGACAA
4	CDSF01000130	CATCAACGTGACAATGGACC	AGTCGAAGTACTGGACCATG
5	CDSF01000112	ACGAAGAGAAAACGTGCAA	AGTACGTGTCGTTGGACTTG
6	CDSF01000133	GAGGTCGACACGTTCTGTAG	CAGGTTCTGGTAGGATGTCA
7	CDSF01000079	GATGGCCTGAACTGCGTT	CTAGTTTGGCAGCGTGATC
8	CDSF01000001	ACGACAGTCGCTGCTCTAT	CAGTAGACGTCGTATGGGATA
9	CDSF01000144	CCCAGATGATTCCGATTAAC	CCTGCCAGTTGTTACCTTG
10	CDSF01000022	AATATGGCGTGGAATGCTG	CGTCATACTGTGGTCTAGGT
11	CDSF01000093	GATCGTCATGTGTGCTGTG	GACCATCATTTGCAGCAAGA
12	CDSF01000141	CAGTTCAGGTAGGTAGCAGA	TAACCCGATCCTGAACCG
13	CDSF01000136	TACATCTCTGGAATGGATCG	AGCAACATCGCTACCTGTT
14	CDSF01000013	GCGGCCATGGACCTCA	TCATGAGGTGTTGGCCG
15	CDSF01000088	GTGTTGGGCTTGATCATCTT	CATTCCGGCAACATTCTACA
16	CDSF01000133	ATGGGTGTATGGTCGTGTC	GTCATCATGCTGGAGAAGG

Genes #1-13 encode proteins with high cysteine contents; Gene #14-16 encode proteins with at least one "RXLR" motif within N-terminal 100 amino acids.

Table 3.3. Relative expression of *Plasmodiophora brassicae* genes over time following inoculation of the *Brassica napus* genotypes '45H29' and 'Westar' with pathotypes 5I and 5X of the pathogen, as determined by quantitative PCR analysis.

Gene #	Accession #	Expression level relative to PRG[a] ($2^{-\Delta CT}$)		Regulation fold change[b] ($2^{-\Delta\Delta CT}$)	Expression level relative to PRG[a] ($2^{-\Delta CT}$)		Regulation fold change[b] ($2^{-\Delta\Delta CT}$)	Expression level relative to PRG[a] ($2^{-\Delta CT}$)		Regulation fold change[b] ($2^{-\Delta\Delta CT}$)
		Samples of 14 dai, P5X on '45H29'	Samples of 21 dai, P5X on '45H29'		Samples of 14 dai P5I on '45H29'	Samples of 21 dai P5I on '45H29'		Samples of 14 dai P5I on 'Westar'	Samples of 21 dai P5I on 'Westar'	
1	CEO96260.1	7.23e-4±0.00*	0.11±0.17	152.14[c]	0.11±0.03	5.73e-4±0.38	0.005[d]	0.003±0.00	0.92±0.00	300.05[e]
2	CEO97299.1	3.03e-3±0.00	0.22±0.33	72.61[c]	0.09±0.01	0.34±0.69	3.78[c]	0.01±0.00	72.8±32.9	6441.17[e]
3	CEP03503.1	1.71e-3±0.00	0.07±0.35	40.94[c]	0.06±0.01	0.02±0.22	0.33[d]	0.002±0.00	0.006±0.00	4.08[e]
4	CEP02521.1	7.36e-4±0.00	0.26±0.17	353.26[c]	4.01±0.01	0.02±0.50	0.005[d]	0.006±0.00	0.18±0.10	28.29[e]
5	CEP01326.1	0.004±0.00	0.06±0.20	15.0[c]	0.08±0.00	0.04±0.37	0.5[d]	0.06±0.03	9.44±4.57	158.20[e]
6	CEP02705.1	0.08±0.01	0.07±0.19	0.88	4.85±0.34	0.25±0.52	0.05[d]	0.83±0.36	0.03±0.00	0.03
7	CEO97429.1	0.03±0.01	0.40±0.64	13.33[c]	0.09±0.01	0.03±0.11	0.33[d]	0.90±0.42	6.06±1.92	6.74[e]
8	CEO94655.1	0.03±0.01	0.16±0.52	5.33[c]	0.08±0.02	0.07±0.55	0.88	5.38±2.34	11.88±1.96	2.21[e]
9	CEO94655.1	0.06±0.01	0.71±0.36	11.83[c]	2.09±0.16	0.19±0.32	0.09[d]	4.99±1.95	10.85±2.92	2.17[e]
10	CEO95403.1	0.04±0.00	0.07±0.25	1.75	0.02±0.00	0.03±0.22	1.5	0.002±0.00	0.14±0.07	66.61
11	CEO99770.1	0.05±0.01	56.17±1.44	1123.40[c]	0.10±0.02	2.18±0.48	21.8[c]	0.47±0.47	42.9±11.9	90.56[e]
12	CEP03113.1	0.003±0.00	0.52±0.02	173.33[c]	0.04±0.01	0.02±0.37	0.5[d]	0.17±0.06	0.68±0.24	4.05[e]
13	CEP02996.1	0.30±0.02	0.19±0.59	0.63	0.68±0.05	1.03±0.30	1.51	1.23±0.41	1.58±0.37	1.26
14	CEO95309.1	0.11±0.03	0.03±0.05	0.27[d]	0.74±0.24	0.02±0.40	0.027[d]	1.40±0.50	1.77±0.38	1.27
15	CEO98976.1	0.001±0.00	0.04±0.31	40.0[c]	0.08±0.02	0.04±0.35	0.5[d]	0.07±0.03	0.63±0.42	9.02[e]
16	CEP02928.1	0.002±0.00	0.03±0.12	15.0[c]	0.03±0.01	0.03±0.64	1	0.50±0.18	1.1±0.31	2.20[e]

a: calculated by delta C_T method, fold difference of expression level = $2^{-\Delta Ct} = 2^{-(Ct\ PTG - Ct\ PRG)}$. PTG: *P. brassicae* target gene; PRG: *P. brassicae* reference gene;
b: calculated by delta-delta C_T method, with regulation level=$2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct\ 21\ dai - \Delta Ct\ 14\ dai)}$;
c: up-regulated genes from 14 dai to 21 dai (fold-difference of regulation level is > 2);
d: down-regulated genes from 14 dai to 21 dai (fold-difference of regulation level is > 2);
e: genes with identical regulating pattern to P5X on '45H29' on combination of P5I and 'Westar' (fold-difference of regulation level is > 2);
*: mean±SD

Table 3.4. Putative identity of 16 genes differentially expressed in *Plasmodiophora brassicae* pathotypes 5 and 5X based on matching entries in the GenBank database of the National Centre for Biotechnology Information.

Gene #	Accession #	E-value	Matching Entry (Accession #)*
1	CDSF01000057	-	-
2	CDSF01000079	4e-46	PbSUNK2 gene (AM411671)
3	CDSF01000145	-	-
4	CDSF01000130	8e-91	ATP binding cassette (KC479018)
5	CDSF01000112	-	-
6	CDSF01000133	-	-
7	CDSF01000079	3.4e-97	Cell surface glycoprotein (AEV89104)
8	CDSF01000001	-	-
9	CDSF01000144	6e-05	Secreted protein (AIG55425)
10	CDSF01000022	-	-
11	CDSF01000093	-	-
12	CDSF01000141	8e-97	hypothetical protein BVRB 020880 (KMS94491)
13	CDSF01000136	4e-05	E3 ubiquitin-protein ligase RHA2B-like (XP _022011114)
14	CDSF01000013	1.6e-58	Pepsin II-4-like (XP _014722413.1)
15	CDSF01000088	2.9e-22	Polysaccharide deacetylase (EPH87004)
16	CDSF01000133	1.6e-05	Zinc finger protein (XP_021363710)

"-": No match with an E-value < 0.0001

*Best match

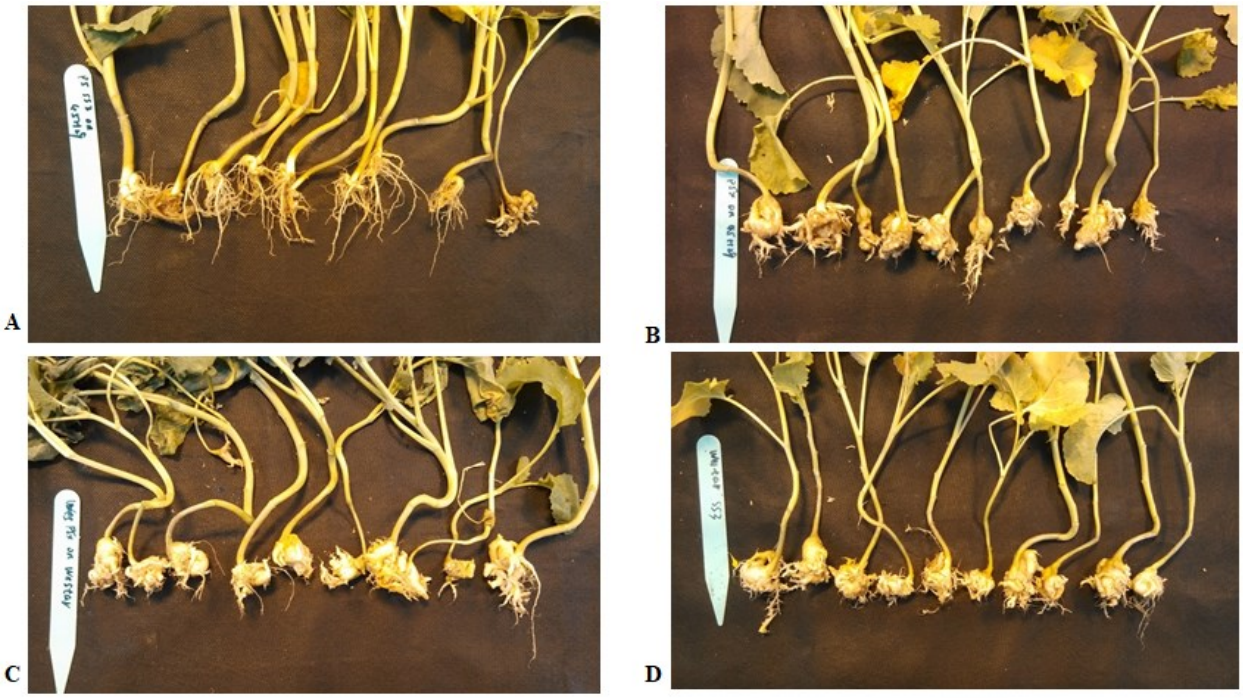

Figure 3.1. Clubroot symptoms in roots of *Brassica napus* '45H29' (A and B) and 'Westar' (C and D) 7 weeks after inoculation with *Plasmodiophora brassicae* pathotypes 5 and 5X. '45H29' was resistant to pathotype 5 (A) and susceptible to pathotype 5X (B). 'Westar' was susceptible to both pathotype 5 (C) and pathotype 5X (D).

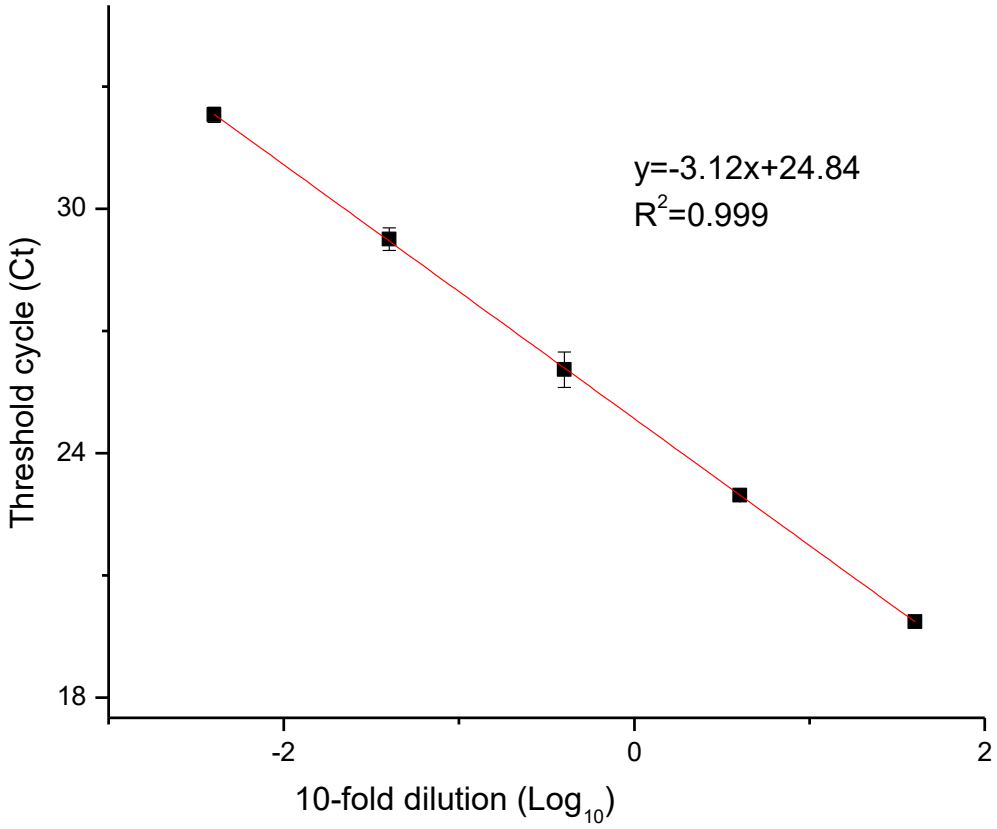

Figure 3.2. Standard curve generated by plotting a 10-fold dilution series of 80 ng of genomic DNA of *Plasmodiophora brassicae* against threshold cycles.

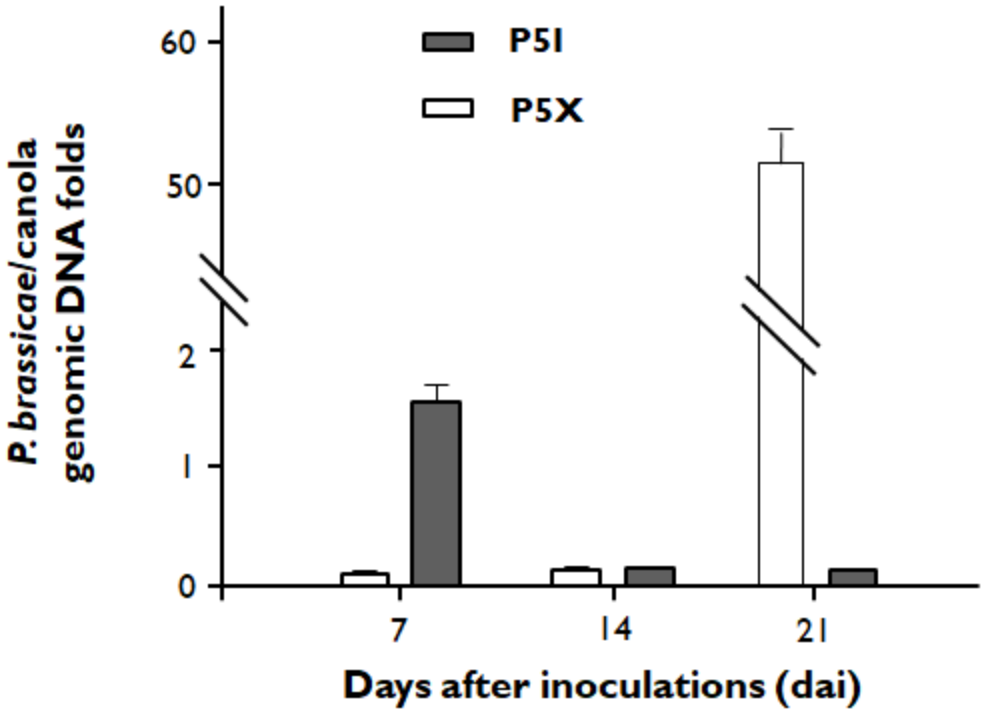

Figure 3.3. Quantification of the relative amount of *Plasmodiophora brassicae* DNA in the canola (*Brassica napus*) genotype '45H29' following inoculation with pathotypes 5I (P5I) or 5X (P5X). The results were normalized by calculating the pathogen/plant biomass ratios in the infected roots following the comparative threshold cycle (Ct) by the delta Ct method, based on the *P. brassicae* reference gene *Pbr018* (Feng et al. 2013) and on the canola reference gene *BnACT2* (Yang et al. 2009). The results shown are from three biological replicates at 7, 14 and 21 days after inoculation. Each replicated experiment included three technical repeats. Error bars indicate the standard error.

Figure 3.4. Heatmaps of gene expression at 14 and 21 days after inoculation (dai) of canola (*Brassica napus*) with *Plasmodiophora brassicae*. Canola '45H29' inoculated with pathotype 5X (**A**). Canola '45H29' inoculated with pathotype 5I (**B**). Canola 'Westar' inoculated with pathotype 5X (**C**).

Chapter 4: QTL mapping and inheritance of clubroot resistance genes derived from *Brassica rapa* subsp. *rapifera* (ECD 02) reveals resistance loci and distorted segregation ratios in three F2 populations of different crosses.

4.1 Introduction

Clubroot is a soilborne disease of the Brassicaceae caused by the obligate parasite *Plasmodiophora brassicae*. Disease development is associated with the formation of large galls on the roots of susceptible plants, which interfere with water and nutrient uptake and lead to significant yield losses in *Brassica* crops (Hwang et al. 2012; Dixon 2014). Yield losses of 20-100% have been reported worlwide including in Canada (Tewari et al. 2005; Rahman et al. 2014), China (Chai et al. 2014), and India (Bhattacharya et al. 2014). The clubroot pathogen survives as resting spores that can persist in the soil for many years (Dixon 2009). Given the longevity of *P. brassicae* in infested soils and the significant economic value of *Brassica* crops, the management of clubroot has been a focus of agricultural researchers for decades. In recent years, clubroot has emerged as an important constraint to canola (*Brassica napus*; oilseed rape) production in western Canada, further increasing interest in this disease (Strelkov and Hwang 2014).

While many strategies have been employed for clubroot control (Hwang et al. 2014), clubroot resistance (CR) is the most effective and environmentally friendly approach to manage the disease (Rahman et al. 2014). The identification of effective resistance is the first step in breeding for this trait, with *Brassica rapa* (2n = AA) considered a superior source of resistance than *B. oleracea* (2n = CC) (Toxopeus et al. 1986; Hirai 2006; Piao et al. 2009). Over the past 20 years, at least 15 CR genes have been identified in *B. rapa*, including *CRa* (Matsumoto et al. 1998), *CRb* (Piao et al. 2004), CRb^{Kato} (Kato et al. 2012, 2013), *CRk* (Matsumoto et al. 2012),

Crr3 (Hirai et al. 2004; Saito et al. 2006), *Rcr2* (Yu et al. 2017), *CRc* (Sakamoto et al. 2008; Matsumoto et al. 2012), *Crr1*, *Crr2*, *Crr4* (Suwabe et al. 2003, 2006), *BraA.Cr.a*, *BraA.Cr.b*, *BraA.Cr.c* (Hirani et al. 2018) and *CrrA5* (Nguyen et al. 2018). Clubroot resistance from *B. rapa* has been introgressed into several European *B. napus* oilseed cultivars, including 'Mendel' and 'Tosca' (Frauen 1999).

In Canada, different *Brasscia* genotypes have been used as resistance donors in the breeding of CR canola/oilseed rape (*Brassica napus*). Fredua-Agyeman and Rahman (2016) reported that the *B. napus* 'Mendel' possesses one dominant CR gene effective against pathotype 3H of *P. brassicae*, as defined on the Canadian Clubroot Differential set (Strelkov et al. 2018). This was the dominant pathotype in Alberta, Canada (Strelkov et al. 2006, 2007), at least prior to the introduction of CR canola cutlivars beginning in 2009 (Strelkov et al. 2018). However, the planting of CR canola in short rotations over large acreages led to the rapid development of new pathotypes of *P. brassicae* (Strelkov et al. 2016; Strelkov et al. 2018), and 'Mendel' resistance was eroded and is not a good choice for breeding cultivars resistant to the new pathotypes. Rutabaga (*Brassica napus* ssp. *napobrassica*) is another potential donor of clubroot resistance, and the inbred rutabaga cultivars 'Brookfield' and 'Polycross' possessed excellent resistance to pathotypes 2, 3, 5, 6, 8 of *P. brassicae* (Hasan and Rahman 2011, 2016). The ratio of resistant (R) to susceptible (S) plants in the F_2 generation derived from crossing 'Brookfield'or 'Polycross' with susceptible *B. napus* lines was 3R:1S, while segregation in the test cross family of the latter deviated from a 1R:1S ratio. This suggested that CR in 'Brookfield' was controlled by a single dominant gene, while resistance in 'Polycross' was more complex (Hasan and Rahman 2011).

With the recent emergence of new pathotypes of *P. brassicae* able to overcome clubroot

resistance in most CR canola culitvars, additional sources of resistance are needed. The *CRa* resistance gene was first detected in the European Clubroot Differential (ECD) 02 (*B. rapa* ssp. *rapifera* line AAbbCC) (Buczacki et al., 1975; Matsumoto et al. 1998). Zhang et al. (2015) reported one marker (i3e4) that was tightly linked to *CRa*. While ECD 02 appears to be resistant to all *P. brassicae* pathotypes identified in Canada to date (Leboldus et al. 2012; Strelkov et al. 2006, 2018), and was used as a resistance source in studies from Japan (Sakamoto et al. 2008; Hayashida et al. 2008), its application in clubroot resistance breeding in Canada has not yet been reported. The objectives of the current study were to introgress clubroot resistance from ECD 02 (male parent) into three susceptible female *B. rapa* genotypes CR 2599, CR 1505 and a *B. napus* genotype CR 1054, and to evaluate the genetic basis of resistance to three isolates of *P. brassicae* representing different pathotypes and identify molecular markers.

4.2 Materials and methods

4.2.1 Development of mapping populations

The parent *B. rapa* subsp. *rapifera* line AAbbCC (ECD 02) was resistant to all 17 *P. brassicae* pathotypes identified in Canada up to 2016 (Strelkov et al. 2018). In contrast, *B. rapa* accessions CR 2599 and CR 1505 ('Emma') and *B. napus* accession CR 1054 ('Westar') were susceptible to these same pathotypes (Fredua-Agyeman et al. 2019) and served as the susceptible parents. ECD 02 is a winter-type while all three susceptible parents are spring-types.

To produce the F_1 plants, crosses were carried out between June 2016 and January 2017 by emasculation followed by hand-pollination, with the plants kept in a growth chamber maintained under an 18 h photoperiod and temperatures of 21/18 ^{o}C (day/night). Vernalization of ECD 02 was carried out as described by Fredua-Agyeman et al. (2019). The susceptible parents were seeded much later to ensure that they flowered around the same time as ECD 02.

Seeding, vernalization and the self-pollination of single F_1 plants to produce F_2 seeds were carried out in a growth chamber, cold room and greenhouses, respectively, at the Crop Diversification Centre North, Alberta Agriculture and Forestry, Edmonton, Alberta from March 2017 to June 2018. The ECD 02 × CR 2599, ECD 02 × CR 1505 and ECD 02 × CR1054 derived F_2 populations will be designated here as Popl#1, Popl#2 and Popl#3, respectively, for convenience.

4.2.2 Pathogen material

Twenty-two *P. brassicae* field and single-spore isolates representing 17 unique pathotypes were used to screen 14 to 24 seedlings of ECD 02. The pathotypes (isolates indicated in parentheses) included: pathotype 2B (F183-14), 2F (SACAN-ss3), 3A (F3-14), 3D (F1-14), 3H (SACAN-ss1 and CDCN#4), 3O (F381-16), 5C (F175-14), 5G (CDCS and CDCN#6), 5I (ORCA-ss4 and CDCN#2), 5K (F10-15), 5L (F-360-13), 5X(LG-1, LG-2 and LG-3), 6M (AbtJE-ss1), 8E (F187-14), 8J (F12-15), 8N (ORCA-ss2) and 8P (UoA#37) (Xue et al. 2008; Strelkov et al. 2016; 2018). Pathotype designations follow Strelkov et al. (2018). The number of isolates used to screen the F_1 seedlings depended on the number of seeds obtained from each cross. Five single-spore isolates (representing pathotypes 2F, 3H, 5I, 6M and 8N) (Xue et al. 2008) and four field isolates (representing pathotypes 2B, 5X (LG-1), 5G (CDCS) and 8J) (Strelkov et al. 2016; 2018) were used to screen 25 F_1 plants of the cross ECD 02 × CR 2599 (Popl#1). Two isolates (pathotypes 5X (LG-1) and 5G (CDCS)) were used to screen two F_1 plants of the cross ECD 02 × CR1505 (Popl#2). Pathotype 3A along with the nine pathotypes used to screen Popl#1 were used to screen 37 F_1 plants of the cross ECD 02 × CR1054 (Popl#3).

F_2 plants of the three populations were screened with pathotypes 3H (predominant in Alberta and represented by the single-spore isolate SACAN-ss1), 5X (the first to overcome

resistance in CR canola and represented by the field isolate LG-1; Strelkov et al. 2016), and 5G (represented by isolate CDCS, one of the most virulent found in an earlier study; Fredua-Agyeman et al. 2019). Thus, segregating ratios in the three F_2 populations (Popl#1, Popl#2 and Popl#3) × pathotypes 3H (SACAN-ss1), 5X (LG-1) and 5G (CDCS) (i.e., nine combinations) were examined.

4.2.3 Inoculum preparation

Isolates of *P. brassicae* were stored as galled roots at -20°C until needed. To prepare resting spore inoculum, the galls were homogenized in sterile distilled water in a Waring LB10G blender (Cole-Parmer) following Fredua-Agyeman et al. (2018). The resulting slurry was passed through eight layers of cheesecloth into a beaker to remove plant debris and other detritus, and the filtrate was collected. The resting spore concentration was estimated using a hemocytometer and adjusted to 1×10^8 spores/mL with sterile distilled water. Inoculum was kept at 4°C and used within 24 h of preparation.

4.2.4 Clubroot assays

Seedlings were geminated in Petri dishes (100 mm × 15 mm) on moistened Whatman no. 1 filter paper for 7 days at room temperature and a 12h photoperiod. Inoculations were carried using the root-dip method (Nieuwhof and Wiering 1961; Strelkov et al. 2006), with additional inoculum added by the pipette method (Lamers and Toxopeus 1977 as cited by Voorrips and Visser 1993). Briefly, the rootlets were dipped into the pathogen resting spore suspension for about 10-20 s and then planted in 8 × 4 flat trays, cell size 7 × 6 × 6 cm, filled with Sunshine Mix #4 Aggregate Plus (Sungro Horticulture, Seba Beach, Alberta, Canada) potting medium.

The seedlings were transferred to a greenhouse maintained at 20-25/15-18°C day/night with a 16 h photoperiod, and watered daily with slightly acidified water (pH ≈ 5.5 - 6.5, adjusted

with HCl). Beginning at 3 weeks after inoculation, the plants were fertilized once a week with 20 N: 20 P: 20 K Classic Fertilizer with micronutrients (Plant Products Brampton, Ontario, Canada). Six weeks after inoculation, the plants were gently removed from the potting medium, washed in water, and assessed for clubroot severity on a 0-3 scale, where: 0 = no galls; 1 = a few small or bead-sized galls on < 1/3 of the roots; 2 = medium galls on 1/3-2/3 of the roots, and 3 = large galls on > 2/3 of the roots (Kuginuki et al. 1999; Xue et al. 2008). The susceptible *B. napus* 'Westar' was included as a positive control in all of the assays.

4.2.5 DNA extraction

Three hundred sixty-eight leaves were collected from 46 F_2 individuals resistant (disease score = 0) and 46 F_2 individuals susceptible (disease score = 3) to each of pathotypes 5X and 5G in Popl#1 and Pop#2. Genomic DNA was extracted from the leaves of the parents and the 368 F_2 individuals using the cetyltrimethyl ammonium bromide (CTAB) method (Sambrook and Russell 2001). The DNA concentration was quantified with a ND-2000c spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and the template DNA diluted to 20-25ng/µL for PCR.

No molecular work was carried out on F_2 plants inoculated with pathotype 3H (for all three populations) because almost all previous genetic mapping studies in Canada have utilized this pathotype. Furthermore, no molecular work was carried out on F_2 plants of Popl#3 inoculated with pathotypes 5X and 5G because the sample size was small. Therefore, molecular analyses were completed on four of the nine plant population/*P. brassicae* pathotype combinations.

4.2.6 PCR and SSR genotyping

PCR amplification was carried out in a 12 µL reaction volume containing 2.5 µL of 5× Taq buffer, 1.0 µL of 25 mM $MgCl_2$, 0.25µL of 10 mM dNTPs mix, 0.25µL of 25 nM of each primer, 0.25µL of 25 nM of fluorescently labelled M1T3 primer, 1.0 µL of 20-25ng DNA template, and 1.25 U of Taq polymerase (Promega, Madison, WI, USA). The PCR cycling conditions consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 58°C for 1 min and 72°C for 1.5 min, and a final elongation step at 72°C for 10 min. Aliquots of the PCR products were analyzed on an ABI PRISM 3730 × 1 DNA analyzer (Applied Biosystems). Amplified products for which the bands from resistant and susceptible plants differed by > 200 bp were separated on 3% agarose gels. The gels were stained with SYBR Safe (Thermo Fisher Scientific, Carlsbad CA, US) and visualized with a Typhoon FLA 9500 Variable Mode Laser Scanner Image Analyzer (GE Healthcare Life Sciences, Mississauga ON, Canada).

Bulk segregant analysis as described by Michelmore et al. (1991) was carried out using 144 PCR-based markers with DNA of the parents and two resistant and two susceptible DNA bulks from each of the two F_2 populations (Popl#1 and Pop#2). The markers included 13 markers spanning the A03 chromosome of *B. rapa*, 65 markers linked to six previously reported clubroot resistant genes: *CRk*, *Crr3*, *CRb*, CRb^{Kato}, *CRa* and *Crr1*, as well as 66 SSR markers designed in this study. The polymorphic markers were used to genotype the parents as well as the 384 F_2 individuals of Popl#1 and Pop#2, and a genotype matrix was constructed for pathotypes 5X and 5G for Popl#1 and Pop#2.

4.2.7 Map construction and QTL analysis

Genetic linkage analysis was performed for each of the four combinations using MAPMAKER/EXP 3.0 (Lincoln et al. 1992). The logarithm of odds (LOD) score was set for a minimum of 3.0 and a recombination fraction (Θ) of 0.40. The Kosambi map function was used

to convert the recombination frequencies into genetic distances (in centimorgans, cM) (Kosambi 1944). Genetic linkage maps were constructed using MapChart (Voorrips 2002). Single Marker Analysis (SMA), Composite Interval Mapping (CIM) and Inclusive Composite Interval Mapping-Additive (ICIM-ADD) methods were performed using 1000 permutations (Churchill and Doerge, 1994) with the genotype data, genetic map and phenotype data using QTL Cartographer (Wang et al. 2011) and ICIM software (Meng et al. 2015).

4.2.8 Statistical analysis

To test for the inheritance of clubroot resistance in the F_2 populations, the phenotypic data from different crosses of the same parents were subjected to Chi-square (χ^2) tests of homogeneity, with data from those found to be sufficiently uniform pooled. The resulting data for each population were subjected to χ^2 goodness-of-fit for different segregation ratios using SAS v. 9.4 (SAS Institute, USA).

4.3 Results

4.3.1 Clubroot assays, genetic crosses and development of F_2 mapping populations

In total, 98% (428 of 435) of ECD 02 plants inoculated with the 22 isolates of *P. brassicae* (representing 17 unique pathotypes) were completely free of clubroot symptoms (disease score = 0) six weeks after inoculation. Sixteen of the 22 isolates did not cause any symptoms at all (disease score = 0) on any plants, while very mild galling (disease score = 1) was observed on a single plant in response to inoculation with each of pathotypes 2B (F183-14), 5I (ORCA-ss4), 5X (F-359-13), 8N (ORCA-ss2) and 8J (F12-15). Two plants of ECD 02 inoculated with pathotype 5G (CDCS, Newell) developed moderate galling (disease score = 2).

Eleven plants of ECD 02 showing absolute resistance (disease score = 0) to pathotype 5G were used as donor parents in crosses with the three susceptible parents CR 2599, CR 1509 and

CR1504. The F_1 plants produced from the three crosses were winter-types and required vernalization to induce flowering. Approximately 76% (25 of 33), 25% (2 of 8) and 9% (37 of 416) of the crosses carried out between ECD 02 and CR 2599, CR 1509 and CR1504, respectively, resulted in siliques. Of the successful crosses, 24% (6 of 25), 50% (1 of 2) and 5% (2 of 37) produced 2-30 good quality rounded seeds per silique. All 64 of the F_1 plants screened with *P. brassicae* (2-10 pathotypes) were resistant to the pathogen (49 disease score = 0, 15 disease score = 1) (Table S4.1). The 49 F_1 plants with a disease score = 0 were self-pollinated.

The development of three F_2 mapping populations from the crosses between ECD 02 and the three susceptible parents is summarized in Figure 4.1. One thousand five hundred and ninety-eight, 710, and 354 F_2 individuals derived from 6, 1 and 2 clubroot resistant F_1 families from Popl#1, Popl#2 and Popl#3, respectively, were evaluated for resistance to the *P. brassicae* pathotypes 3H (SACAN-ss1), 5X (LG-1) and 5G (CDCS). The frequency distribution of disease scores to the three pathotypes in the three F_2 populations is presented in Figure 4.2. Seven of the nine distributions, with the exceptions of Polpl#3-5X and Popl#3-5G, were bimodal, suggesting oligogenic or polygenic resistance to clubroot in the three F_2 populations.

4.3.2 Phenotypic variation in F_2 mapping subpopulations

The χ^2 tests of homogeneity indicated that the phenotypic data from the 534 F_2 plants of Popl#1 inoculated with pathotype 3H and the 524 F_2 plants inoculated with pathotype 5X were significantly different ($P < 0.00001$) and hence could not be pooled (Table S4.2 and S4.3). In contrast, no significant differences were found in the phenotypic data from the 490 F_2 plants of Popl#1 inoculated with pathotype 5G (Table S4.4).

In the case of Popl#2, the 227, 244 and 239 F_2 plants inoculated with pathotypes 3H, 5X, and 5G, respectively, were produced by self-pollination of a single F_1 family from the cross ECD

02 × CR 1505 and hence, no χ^2 tests of homogeneity were conducted. Similarly, the phenotypic data from the 73 F_2 plants of Popl#3 inoculated with pathotype 5G were not subjected to χ^2 tests of homogeneity because seeds were obtained from only one of the two F_1 families from the cross ECD 02 × CR1054. Instead, the F_2 data for Popl#2 inoculated with 3 pathotypes and Popl#3 inoculated with pathotype 5G were tested directly for χ^2 goodness-of-fit for different segregation ratios.

The remaining F_2 plants from Popl#3, which were inoculated with pathotypes 3H and 5X, were produced from the self-pollination of two F_1 families of the cross ECD 02 × CR1054. The χ^2 tests of homogeneity suggested the phenotypic data from the 109 plants screened with pathotype 3H were not significantly different (P = 0.0625 and 0.7692) (Table S4.5) and therefore could be pooled. In contrast, the 172 F_2 plants inoculated with pathotype 5X could not be pooled because 25-50% of the calculated expected (E) values were < 5 (a requirement for conducting χ^2 tests of homogeneity) (Table S4.6).

4.3.3 Inheritance pattern of clubroot resistance derived from ECD 02

The segregation analysis was carried out in two ways. The first considered only those plants with a disease score = 0 as resistant (R), and all others as susceptible (S) (Tables 2, 3, 4). The second considered plants with disease scores = 0 or 1 as R, and those with disease scores = 2 or 3 as S (Tables 2, 3, 4). The χ^2 goodness of fit test showed that the segregation of clubroot resistance in the F_2 populations largely deviated from the expected Mendelian segregation ratios of 3:1, 15:1 and 63:1 for resistance controlled by a single, two or three dominant genes, respectively. Instead, deviations from the normal ratios, such as those observed in the event of non-allelic or linked interactions, were obtained.

Sixty-eight and 50 plants of two of the six F_2 families from Popl#1 inoculated with

pathotype 3H fit the 3R:1S segregation ratio in addition to the 9R:7S and or 11R:5S ratios. This suggested one-gene-control of clubroot resistance (Table 4.1). Thirty-four, 151, 176 and 55 F_2 plants of the remaining four families exhibited segregation ratios of 9R:7S, 5R:11S and 11R:5S to pathotype 3H, suggesting the introgression of two CR genes from ECD 02 (Table 4.1). Seventy-six, 47, 142, 163, 56 and 40 plants of the six F_2 families of Popl#1 inoculated with pathotype 5X showed segregation ratios of 9R:7S, 5R:11S and 1R:15S (Table 4.1). Finally, all 490 (pooled data for the six F_2 families) F_2 plants of Popl#1 inoculated with pathotype 5G fit a 5R:11S segregation ratio (Table 4.1).

In the case of Popl# 2, the 227 F_2 plants (derived from a single cross) inoculated with pathotype 3H gave a good fit for 9R:7S (Table 4.2). Three ratios, 3R:1S, 9R:7S and 11R:5S, could fit the data obtained with the 244 F_2 plants inoculated with pathotype 5X. Ratios of 9R:7S and 11R:5S segregation ratios could fit the data for the 239 F_2 plants inoculated with pathotype 5G (Table 4.2).

The 109 F_2 plants of Popl#3 (pooled data for the two F_2 families) inoculated with pathotype 3H exhibited both the 3R:1S ratio expected for one-gene-control of clubroot resistance to this pathotype and ratios of 5R:11S and 11R:5S obtained for deviations from the Mendelian segregation ratios for two-gene-control (Table 4.3). Similarly, the 73 plants from Popl#3 inoculated with pathotype 5G fit ratios of 3R:1S, 9R:7S and 11R:5S, as was observed for the plants inoculated with pathotype 3H (Table 4.3). In contrast, the 144 and 28 plants inoculated with pathotype 5X were mostly susceptible. The segregation fit 5R:11S and 1R:15S ratios, which are consistent with a two-gene-control of resistance, and a 1R:63S ratio, which is consistent with three-gene control of clubroot resistance.

4.3.4 Linkage analyses and QTL mapping

Of 144 PCR-based markers screened by bulk segregant analysis, 49 markers detected polymorphism between the parents of Popl#1 (i.e., ECD 02 and CR 2599), while 45 markers detected polymorphism between the parents of Popl#2 (i.e., ECD 02 and CR 1505). Twenty-seven of the 49 and 23 of the 45 markers detected polymorphisms in Popl#1 and Popl#2, respectively. Sixteen of these markers detected polymorphism in the two F_2 populations, while 11 and 7 markers detected polymorphism only in Pop#1 and Popl#2, respectively. Table 4.4 provides the sequence information and origin of the 34 (16 + 11 + 7) polymorphic markers used to genotype the two F_2 populations.

At a LOD threshold of 3.0 and a recombination fraction (Θ) of 0.40, 14 of the 25 A03 chromosome markers used to screen the F_2 plants of Popl#1 inoculated with pathotype 5X were linked, while 11 of the A03 chromosome markers remained unlinked. Two QTLs for resistance to pathotype 5X were detected on chromosome A03of *B. napus* based on the ICIM-ADD method. The SSR markers KB59N05 and B4732 bordered the first QTL (LOD score=3.6, located between 13.4-21.3 cM (Figure 4.3) and ≈ 24274312 to 24348056 nt on the physical map of *B. napus*). The SSR markers BGA06 and KB29N19 bordered the second QTL (LOD score=15.9, located between 33.8-41.2 cM (Figure 4.3) and ≈ 24426905 to 24637310 nt on the physical map of *B. napus*). These two QTLs explained 4.5% and 51.0% of the phenotypic variance, respectively. Single Marker Analysis (SMA) with the IciMapping software indicated that the SCAR marker GC2360-1 had the highest LOD and PVE (phenotypic variation explanation) scores of 14.2 and 48.9%, respectively, followed by marker KB59N06 with LOD and PVE scores of 5.7 and 23.8%, respectively. Overall, the linked markers explained 55.4% and 72.7% of the phenotypic variance based on the ICIM-ADD and SMA methods, respectively.

Similarly, in the F_2 plants from Popl#1 inoculated with pathotype 5G, 18 of the 25

markers on the A03 chromosome were linked, while three of the markers remained unlinked. The ICIM-ADD method identified two QTLs from the A03 chromosome of *B. napus* for resistance to pathotype 5G. The SSR markers KB59N06 and B4732 bordered the first QTL (LOD score =17.0, located between 32.7-46.6 cM (Figure 4.4) and ≈ 24262454 to 24348056 nt on the physical map of *B. napus*). The SSR markers CRaJY and BGB41 flanked the second QTL (LOD score =14.0, located between 76.8-92.0 cM (Figure 4.4) and ≈ 24557499 to 24579679 nt on the physical map of *B. napus*). These two QTLs explained 23.3% and 21.8% of the phenotypic variance, respectively. The SMA with the IciMapping software indiciated that SSR marker KB59N03 had the most significant association, with LOD and PVE scores of 6.8 and 27.4%, respectively. This was followed by marker GC2360-1 with LOD and PVE scores of 6.3 and 25.7%, respectively. In general, based on the IciMapping software and using the ICIM-ADD and SMA methods, the PVE by the linked markers was 45.2% and 53.1%, respectively.

The linkage analysis indicated that almost all (at least 19 of the 21) of the polymorphic markers on the A03 chromosome were linked for F_2 plants of Popl#2 inoculated with pathotypes 5X or 5G. However, only the second QTL for resistance was detected by the ICIM-ADD method in these plants following inoculation with pathotypes 5X (LOD score=16.7, Figure 4.5) or 5G (LOD score=17.4, Figure 4.6), respectively. This QTL was found in the interval between SSR markers CRaJY and KB29N19, in the genomic region located from approximately 24557499 to 24637310 nt on the physical map of *B. napus*. The percentage of phenotypic variance explained by the ICIM-ADD method was 35.6% and 32.5% for the F_2 plants inoculated with pathotypes 5X or 5G, respectively. The SMA with the IciMapping software indicated that SSR marker GC2360-1 had the most significant association in response to infection by *P. brassicae* pathotypes 5X and 5G. The LOD scores for the SMA were 6.6 and 4.7 for the F_2 plants

inoculated with pathotypes 5X or 5G, respectively, while the PVE scores were 27.0% and 29.3 %.

The LOD profiles and PVE determined by the CIM and SMA methods implemented in WinQTL Cartographer for the F_2 plants from Popl#1 and Popl#2 inoculated with pathotypes 5X or 5G showed the same pattern as those determined with the IciMapping software. For example, in Popl#1, the LOD score values for the first and second QTLs for pathotypes 5X were 4.2 and 8.2, respectively, with the CIM method compared with 3.6 and 15.9 by the ICIM-ADD method. The LOD score values for first and second QTLs for pathotype 5G were 21.3 and 6.5, respectively, with the CIM method compared with 17.0 and 14.0 by the ICIM-ADD method. In the case of Popl#2, the LOD scores for the second QTL were 6.2 and 6.9 for pathotypes 5X and 5G, respectively, with the CIM method compared with 16.7 and 17.4 by the ICIM-ADD method. Thus, the LOD scores determined with the ICIM software were in general about twice as high as the values obtained with WinQTL cartographer. The additive effects detected with IciMapping and WinQTL cartographer software were positive for the QTLs, suggesting that the alleles conferring clubroot resistance were derived from the resistant parent ECD 02.

In the case of the two co-segregating markers on the A08 chromosome, recombination between the BRMS-088 allele and clubroot resistance in the F_2 plants of Popl#1 inoculated with pathotype 5X or 5G was 10.4% and 15.6%, respectively. Similarly, recombination between marker allele and phenotype was 7.3% and 25.0% in the F_2 plants of Popl#2 inoculated with pathotype 5X or 5G, respectively. Recombination between the SSR marker A08-5021 allele and clubroot resistance in the F_2 plants of Popl#1 inoculated with pathotypes 5X or 5G was 9.4% and 28.1%, respectively, while the recombination in Popl#2 was 7.3% and 11.5%, respectively. Thus, recombination between the two A08 markers and clubroot resistance to pathotype 5X was much

smaller (7.3-10.4%) relative to that for resistance to pathotype 5G (11.5-28.1%). Epistatic interaction (Q × Q) analysis indicated the PVE at levels ranging from 0.0 to 17.6% for the A03 ×A03 QTLs, 35.6 to 53.4% for the A03 ×A08 QTLs, and 25.8 to 37.3% for the A08 ×A08 QTLs. The results of the epistatic QTL analysis suggest that genes from both the A03 and the A08 chromosomes are needed for resistance to be effective.

4.4 Discussion

Clubroot is widespread in many of the *Brassica* growing areas of the world. Both quantitative and qualitative resistance genes are present in *Brassica* crops (Piao et al., 2009). The overcome of single dominant CR genes in commercial canola cultivars and the emergence of new virulent isolates of *P. brassicae* have been reported in Canada (Strelkov et al. 2016; 2018) and Europe (Oxley 2007, Diederichsen et al. 2014; Wallenhammar et al. 2014; Zamani-Noor 2017). The erosion of the effectiveness of CR genes has also occurred in cruciferous vegetables in Asia (Bhattacharya et al. 2014, Chai et. al 2014). The elevated infection in clubroot-resistance cultivars and volunteers would lead to increased spore load of the pathogen in the soil (Zamani-Noor and Rodemann, 2017). Hence, clubroot remains a huge problem and poses by far the most significant threat to cruciferous crop production worldwide.

One of the strategies to combat clubroot caused by the many pathotypes of *P. brassicae* is to deploy cultivars with multiple CR genes. The inheritance of different encoding genes provide a buffer when one resistance mechanism is overcome (Lagudah 2011). However, the combined effects of inheriting gene combinations seems to be very complex. For example, genetic analyses in durum wheat (*Triticum turgidum* L. ssp. *durum*) indicated strong additive effects of *Lr34*/*Yr18* gene combination to leaf rust, stripe rust and powdery mildew compared to the resistance effects of the *Lr46*/*Yr29* gene combination to the three diseases (Lillemo et al. 2008). Therefore, in

canola different gene combinations is expected to confer different levels of resistance to clubroot.

In this study, the inheritance of multiple CR genes was examined in F_2 plants derived from the crossing between *Brassica rapa* L. spp. *rapifera* line ECD 02 (turnip) (the resistant parent) and two *B. rapa* accessions and one *B. napus* accession (the susceptible parents). The fact that all the F_1 plants from the above crosses were highly resistant against pathotypes 3H, 5X and 5G suggested that multiple CR genes were successfully introgressed from ECD 02. The F_1 mean of all crosses were not significantly different from the resistant parent ECD 02 but were significantly different from the mean measurements of all three susceptible parents. These results indicate the complete dominance genes controlling clubroot resistance genes came from ECD 02. The segregation of F_2 plants for clubroot resistance (Tables 4.1, 4.2 and 4.3) and the fact that the F_2 means of all crosses were significantly different from the mean measurements of the both parents suggested that the genetic variance consisted of both additive and dominance effects. The 3R:1S ratio was consistent with the inheritance of a trait controlled by a single dominant major gene while the 9R:7S, 5R:11S, 11R:5S and 1R:15S ratios are modifications of the 15R:1S segregation ratio expected for a trait controlled by two dominant major genes.

The distorted segregation ratios suggested that the resistance genes were on different chromosomes (Hayman and Mather, 1955). The 9R:7S ratio confirms the existence of two genes with duplicate recessive epistasis, suggesting that the dominant allele at the two loci were necessary to control the clubroot caused by pathotypes 5X and 5G. In other words, individuals with double recessive at either locus or both loci were susceptible. The 5R:11S, 11R:5S and 1R:15S indicated two-gene control of clubroot resistance with digenic additive epistasis or quantitative control of the resistance. In the current study, most of the segregation fitted the two-

gene model although segregation patterns of 3R:1S and 1R:63S were also possible. However, the mapping population was of the F_2 generation and hence it is not clear if the preponderance of non-allelic gene effects will alter at advanced generations. Pioneering studies based on segregation ratios suggested that at least two dominant major genes (originally designated A and C) controlled clubroot resistance in ECD 02 (Wit, 1964; Buczacki et al., 1975). Therefore, the results of the greenhouse screening work were in agreement with the published literature.

B. rapa is a very rich source of dominant major CR genes and hence have been used as resistance donors in many breeding programs around the world. Published CR genes derived from *B. rapa* include *Crr1*, *Crr2*, *Crr3*, *Crr4*, *CRa*, *CRb*, CRb^{Kato}, *CRc*, *CRd*, *CRk*, *Rcr1*, *BraA.Cr.a*, *BraA.Cr.b*, *BraA.Cr.c* and *CrrA5* (Matsumoto et al. 1998, 2012; Suwabe et al. 2003, 2006; Hirai et al. 2004, 2006; Piao et al. 2004; Saito et al. 2006; Sakamoto et al. 2008; Kato et al. 2012, 2013; Ueno et al. 2012; Hatakeyama et al. 2013; Zhang et al. 2014; Chu et al. 2014; Yu et al. 2016; Pang et al. 2018; Hirani et al. 2018; Nguyen et al. 2018). Most of the previous genetic mapping studies in Canada and Europe relied on single gene resistance resources as donors (Diederichsen et al. 2009; Chu et al 2014; Fredua-Agyeman and Rahman 2016; Hassan and Rahman 2016; Yu et al 2016). Matsumoto et al. (1998) reported that ECD 02 possessed the *CRa* gene. Ueno et al. (2012) mapped the *CRa* gene to a genomic region on the A03 chromosome of *B. rapa* between the SCAR markers GC2360 and GC1680. Hirani et al. (2018) reported that ECD 02 possessed another clubroot resistant gene, *BraA.CR.b*, on the A08 chromosome of *B. rapa* that could be similar to the *Crr1* gene previously reported by Hatakeyama et al. (2013). Therefore, the genomic regions identified in this study were consistent with the results of previous studies that mapped the clubroot resistant loci derived from ECD 02 to the A03 and the A08 chromosomes.

In spite of the substantial contribution of non-additive effects to the variation of complex traits, gene effects controlling clubroot resistance has not been studied. Yu et al. (2017) reported that the *Rcr8* gene on the A02 chromosome and *Rcr9* gene on the A08 chromosome conferred resistance against pathotype 5X. However, the induced resistance to pathotype 5X was not correlated with the resistance to pathotypes 2F, 3H, 5I, 6M and 8N conferred by the *Rcr4* gene on the A02 chromosome. On the other hand, our study shows that resistance conferred by *CRa*/*CRb*^{Kato} gene(s) on the A03 and the *Crr1* gene on the A08 chromosome also conferred resistance to pathotype 3H. In addition, Yu et al. (2017) did not show the different interactions involving the multiple (*Rcr4*, *Rcr8* and *Rcr9*) resistance genes they identified. To the best of our knowledge, our study is the first report that demonstrates that the two CR genes in ECD 02 interact in a non-additive manner to confer resistance to clubroot. Such non-allelic interactions of multiple genes have been reported in the response of many plants to fungi, bacteria, virus and insect attack. For example, barrel clover (*Medicana truncatula*) to aphid attack (Kamphius et al. 2019); mungbean (*Vigna radiate*) to mungbean yellow mosaic virus attack (Akbar et al. 2017); Soybean (*Glycine max*) to rust infection (Pierozzi et. al 2008) and cotton (*Gossypium hirsutum*) to insect pest and virus attacks (Ahuja et al. 2007).

In summary, clubroot tests, linkage analysis and QTL mapping carried out in this study demonstrated that the *CRa/CRb*^{Kato} and the *Crr1* genes on the A03 and the A08 chromosomes of ECD 02 interact in a non-allelic manner to confer clubroot resistance against pathotypes 5X and 5G. Based on the QTL analysis, the genetic control against virulent *P. brassicae* pathotypes may also involve additional genes modulating the action of the two major genes. The presence of at least the two dominant genes complementing each other might explain why ECD 02 confers strong and highly stable qualitative resistance to many *P. brassicae* pathotypes from around the

world. Knowledge of gene effects controlling clubroot resistance offers the possibility of exploiting ECD 02 resistance for the breeding of clubroot resistant canola cultivars and cruciferous vegetables. In addition, the genomic regions identified in this study will provide additional resources for marker-assisted selection in Brassica breeding programs.

Table 4.1. Segregation ratios for resistance to clubroot in an ECD 02 × CR 2599 derived F_2 population under greenhouse conditions.

Popl#/ Fam	Pathotype	Total No. of F_2 (Score=No. plants)	Tested ratio	DF	Observed (disease score)[1]		Test of statistics [1]		Observed (disease score)[2]		Test of statistics [2]	
					R (0)	S (1+2+3)	χ^2	Prob	R (0 +1)	S (2+3)	χ^2	Prob
Popl#1	3H	34	3R:1S	1	10	24	37.7	< 0.00001	14	20	20.7	< 0.00001
Fam 1		(Score 0 = 10)	9R:7S	1			10.0	0.0016			3.1	**0.0764***
		(Score 1 = 4)	5R:11S	1			0.1	**0.8231***			1.6	**0.2117***
		(Score 2 = 2)	11R:5S	1			24.5	< 0.00001			12.0	0.0005
		(Score 3 = 18)	15R:1S	1			240.2	< 0.00001			160.4	< 0.00001
			1R:15S	1			31.1	< 0.00001			70.8	< 0.00001
			63R:1S	1			1053.2	< 0.00001			724.8	< 0.00001
			1R:63S	1			171.2	< 0.00001			346.6	< 0.00001
Popl#1	3H	68	3R:1S	1	39	29	11.3	0.0008	57	11	2.8	**0.0931***
Fam 2		(Score 0 = 39)	9R:7S	1			0.03	**0.8625***			21.0	< 0.00001
		(Score 1 = 18)	5R:11S	1			21.6	< 0.00001			87.5	< 0.00001
		(Score 2 = 2)	11R:5S	1			4.1	**0.0429***			7.2	0.0073
		(Score 3 = 9)	15R:1S	1			153.7	< 0.00001			11.4	0.0007
			1R:15S	1			303.1	< 0.00001			698.4	< 0.00001
			63R:1S	1			746.3	< 0.00001			94.4	< 0.00001
			1R:63S	1			1375.2	< 0.00001			2990.7	< 0.00001
Popl#1	3H	151	3R:1S	1	34	117	221.8	< 0.00001	38	113	200.0	< 0.00001
Fam 3		(Score 0 = 34)	9R:7S	1			69.8	< 0.00001			59.3	< 0.00001
		(Score 1 = 4)	5R:11S	1			5.4	0.020137			2.6	**0.1069***
		(Score 2 = 11)	11R:5S	1			150.2	< 0.00001			133.5	< 0.00001
		(Score 3 = 102)	15R:1S	1			1307.7	< 0.00001			1212.2	< 0.00001
			1R:15S	1			68.2	< 0.00001			92.21	< 0.00001
			63R:1S	1			5658.7	< 0.00001			5270.7	< 0.00001
			1R:63S	1			430.2	< 0.00001			546.0	< 0.00001
Popl#1	3H	176	3R:1S	1	65	111	136.0	< 0.00001	77	99	91.7	< 0.00001
Fam 4		(Score 0 = 65)	9R:7S	1			26.7	< 0.00001			11.2	0.0008
		(Score 1 = 12)	5R:11S	1			2.6	**0.1069***			12.8	0.0003
		(Score 2 = 9)	11R:5S	1			82.9	< 0.00001			51.2	< 0.00001
		(Score 3 = 90)	15R:1S	1			969.7	< 0.00001			750.9	< 0.00001
			1R:15S	1			282.8	< 0.00001			422.4	< 0.00001
			63R:1S	1			4328.8	< 0.00001			3422.2	< 0.00001
			1R:63S	1			1429.9	< 0.00001			2034.7	< 0.00001

* Observed segregation ratio not significantly different from the tested ratio at $P \leq 0.05$.

Table 4.1. Segregation ratios for resistance to clubroot in an ECD 02 × CR 2599 derived F_2 population (continued).

Popl#/ Fam	Pathotype	Total No. of F_2 (Score=No. plants)	Tested ratio	DF	Observed (disease score)[1]		Test of statistics [1]		Observed (disease score)[2]		Test of statistics [2]	
					R (0)	S (1+2+3)	χ^2	Prob	R (0 +1)	S (2+3)	χ^2	Prob
Popl#1 Fam 5	3H	50	3R:1S	1	39	11	0.2	**0.6547***	40	10	0.7	**0.4131***
		(Score 0 = 39)	9R:7S	1			9.6	0.0019			11.5	0.0007
		(Score 1 = 1)	5R:11S	1			50.9	< 0.00001			55.3	< 0.00001
		(Score 2 = 0)	11R:5S	1			2.0	**0.1573***			2.9	**0.0859***
		(Score 3 = 10)	15R:1S	1			21.2	< 0.00001			16.1	0.0001
			1R:15S	1			439.3	< 0.00001			464.1	< 0.00001
			63R:1S	1			135.8	< 0.00001			110.5	< 0.00001
			1R:63S	1			1898.6	< 0.00001			1999.3	< 0.00001
Popl#1 Fam 6	3H	55	3R:1S	1	20	35	43.8	< 0.00001	27	28	19.7	< 0.00001
		(Score 0 = 20)	9R:7S	1			8.8	0.0030			1.2	**0.2835***
		(Score 1 = 7)	5R:11S	1			0.7	**0.4028***			8.2	0.0043
		(Score 2 = 3)	11R:5S	1			26.9	< 0.00001			9.9	0.0017
		(Score 3 = 25)	15R:1S	1			309.1	< 0.00001			187.2	< 0.00001
			1R:15S	1			85.1	< 0.00001			172.3	< 0.00001
			63R:1S	1			1377.8	< 0.00001			870.8	< 0.00001
			1R:63S	1			432.6	< 0.00001			807.2	< 0.00001
Popl#1 Fam 1	5X	76	3R:1S	1	25	51	71.9	< 0.00001	44	32	11.9	0.0006
		(Score 0 = 25)	9R:7S	1			16.8	0.00004			0.1	**0.7773***
		(Score 1 = 19)	5R:11S	1			0.1	**0.7518***			25.1	< 0.00001
		(Score 2 = 4)	11R:5S	1			45.5	< 0.00001			4.2	0.0411
		(Score 3 = 28)	15R:1S	1			480.4	< 0.00001			166.8	< 0.00001
			1R:15S	1			92.1	< 0.00001			346.0	< 0.00001
			63R:1S	1			2122.7	< 0.00001			812.2	< 0.00001
			1R:63S	1			484.5	< 0.00001			1567.0	< 0.00001
Popl#1 Fam 2	5X	47	3R:1S	1	4	43	110.8	< 0.00001	14	33	51.2	< 0.00001
		(Score 0 = 4)	9R:7S	1			43.53	< 0.00001			13.4	0.0003
		(Score 1 = 10)	5R:11S	1			11.3	0.0008			0.05	**0.8231***
		(Score 2 = 3)	11R:5S	1			79.4	< 0.00001			33.2	< 0.00001
		(Score 3 = 30)	15R:1S	1			582.8	< 0.00001			328.2	< 0.00001
			1R:15S	1			0.4	**0.5271***			44.4	< 0.00001
			63R:1S	1			2471.1	< 0.00001			1440.1	< 0.00001
			1R:63S	1			14.7	0.0001			243.1	< 0.00001

* Observed segregation ratio not significantly different from the tested ratio at $P \leq 0.05$.

Table 4.1. Segregation ratios for resistance to clubroot in an ECD 02 × CR 2599 derived F_2 population (continued).

Popl#/ Fam	Pathotype	Total No. of F_2 (Score=No. plants)	Tested ratio	DF	Observed (disease score)[1]		Test of statistics [1]		Observed (disease score)[2]		Test of statistics [2]	
					R (0)	S (1+2+3)	χ^2	Prob	R (0 +1)	S (2+3)	χ^2	Prob
Popl#1	5X	142	3R:1S	1	59	83	84.7	< 0.00001	86	56	15.8	0.00007
Fam 3		(Score 0 = 59)	9R:7S	1			12.5	0.0004			1.1	**0.3009***
		(Score 1 = 27)	5R:11S	1			7.0	0.0082			56.8	< 0.00001
		(Score 2 = 6)	11R:5S	1			48.9	< 0.00001			4.4	0.0353
		(Score 3 = 50)	15R:1S	1			660.4	< 0.00001			266.9	< 0.00001
			1R:15S	1			302.0	< 0.00001			714.9	< 0.00001
			63R:1S	1			2987.8	< 0.00001			1324.3	< 0.00001
			1R:63S	1			1474.8	< 0.00001			3212.0	< 0.00001
Popl#1	5X	163	3R:1S	1	47	116	185.3	< 0.00001	77	86	67.0	< 0.00001
Fam 4		(Score 0 = 47)	9R:7S	1			49.8	< 0.00001			5.4	0.0204
		(Score 1 = 30)	5R:11S	1			0.4	**0.5271***			19.4	0.00001
		(Score 2 = 13)	11R:5S	1			120.9	< 0.00001			35.1	< 0.00001
		(Score 3 = 73)	15R:1S	1			1172.3	< 0.00001			601.8	< 0.00001
			1R:15S	1			141.9	< 0.00001			467.4	< 0.00001
			63R:1S	1			5134.1	< 0.00001			2777.9	< 0.00001
			1R:63S	1			787.0	< 0.00001			2209.2	< 0.00001
Popl#1	5X	56	3R:1S	1	2	54	152.4	< 0.00001	9	47	103.7	< 0.00001
Fam 5		(Score 0 = 2)	9R:7S	1			63.1	< 0.00001			36.7	< 0.00001
		(Score 1 = 7)	5R:11S	1			20.0	< 0.00001			6.0	0.0142
		(Score 2 = 7)	11R:5S	1			110.7	< 0.00001			72.3	< 0.00001
		(Score 3 = 40)	15R:1S	1			777.2	< 0.00001			576.7	< 0.00001
			1R:15S	1			0.7	**0.4028***			9.2	0.0024
			63R:1S	1			3276.6	< 0.00001			2470.0	< 0.00001
			1R:63S	1			1.4	**0.2367***			76.4	< 0.00001
Popl#1	5X	40	3R:1S	1	7	33	70.5	< 0.00001	12	28	43.2	< 0.00001
Fam 6		(Score 0 = 7)	9R:7S	1			24.4	< 0.00001			11.2	0.0008
		(Score 1 = 5)	5R:11S	1			3.5	**0.0614***			0.03	**0.8625***
		(Score 2 = 1)	11R:5S	1			48.9	< 0.00001			28.0	< 0.00001
		(Score 3 = 27)	15R:1S	1			396.9	< 0.00001			277.4	< 0.00001
			1R:15S	1			8.6	0.0034			38.5	< 0.00001
			63R:1S	1			1703.6	< 0.00001			1218.1	< 0.00001
			1R:63S	1			65.9	< 0.00001			210.0	< 0.00001

* Observed segregation ratio not significantly different from the tested ratio at $P \leq 0.05$.

Table 4.1. Segregation ratios for resistance to clubroot in an ECD 02 × CR 2599 derived F_2 population (continued).

Popl#/ Fam	Pathotype	Total No. of F_2 (Score=No. plants)	Tested ratio	DF	Observed (disease score)[1]		Test of statistics [1]		Observed (disease score)[2]		Test of statistics [2]	
					R (0)	S (1+2+3)	χ^2	Prob	R (0 +1)	S (2+3)	χ^2	Prob
Popl#1	5G	490	3R:1S	1	124	366	645.4	< 0.00001	164	326	450.8	< 0.00001
Pooled		(Score 0 = 124)	9R:7S	1			190.7	< 0.00001			103.3	< 0.00001
			5R:11S	1			8.1	0.0044			1.1	**0.2899***
		(Score 1 = 40)	11R:5S	1			430.5	< 0.00001			283.9	< 0.00001
		(Score 2 = 33)	15R:1S	1			3917.5	< 0.00001			3038.8	< 0.00001
			1R:15S	1			303.7	< 0.00001			619.6	< 0.00001
		(Score 3 = 293)	63R:1S	1			17038.2	< 0.00001			13446.7	< 0.00001
			1R:63S	1			1792.8	< 0.00001			3239.2	< 0.00001

* Observed segregation ratio not significantly different from the tested ratio at $P \leq 0.05$.

Table 4.2. Segregation ratios for resistance to clubroot in an ECD 02 × CR 1505 derived F_2 population under greenhouse conditions.

Popl/ Fam	Pathotype	Total No. of F_2 (Score=No. plants)	Tested ratio	DF	Observed (disease score)[1]		Test of statistics [1]		Observed (disease score)[2]		Test of statistics [2]	
					R (0)	S (1+2+3)	χ^2	Prob	R (0 +1)	S (2+3)	χ^2	Prob
Popl#2	3H	227	3R:1S	1	104	123	103.1	< 0.00001	130	97	38.1	< 0.00001
Fam 1		(Score 0 = 104)	9R:7S	1			10.0	0.0015			0.1	**0.7518***
		(Score 1 = 26)	5R:11S	1			22.4	< 0.00001			71.5	< 0.00001
		(Score 2 = 20)	11R:5S	1			55.6	< 0.00001			13.9	0.00019
		(Score 3 = 77)	15R:1S	1			890.2	< 0.00001			515.6	< 0.00001
			1R:15S	1			606.5	< 0.00001			1008.4	< 0.00001
			63R:1S	1			4086.8	< 0.00001			2501.4	< 0.00001
			1R:63S	1			2887.7	< 0.00001			4577.0	< 0.00001
Popl#2	5X	244	3R:1S	1	143	101	35.0	< 0.00001	178	66	0.5	**0.4583***
Fam 1		(Score 0 = 143)	9R:7S	1			0.6	**0.4583***			27.7	< 0.00001
		(Score 1 = 35)	5R:11S	1			85.0	< 0.00001			197.5	< 0.00001
		(Score 2 = 12)	11R:5S	1			11.7	0.0006			2.0	**0.1573***
		(Score 3 = 54)	15R:1S	1			514.3	< 0.00001			180.1	< 0.00001
			1R:15S	1			1141.5	< 0.00001			1852.7	< 0.00001
			63R:1S	1			2516.8	< 0.00001			1030.5	< 0.00001
			1R:63S	1			5159.0	< 0.00001			8081.2	< 0.00001
Popl#2	5G	239	3R:1S	1	137	102	39.8	< 0.00001	162	77	6.6	0.0100
Fam 1		(Score 0 = 137)	9R:7S	1			0.1	**0.7401***			12.9	0.0003
		(Score 1 = 25)	5R:11S	1			75.6	< 0.00001			148.5	< 0.00001
		(Score 2 = 16)	11R:5S	1			14.5	0.0001			0.1	**0.7518***
		(Score 3 = 61)	15R:1S	1			541.3	< 0.00001			275.0	< 0.00001
			1R:15S	1			1063.9	< 0.00001			1544.4	< 0.00001
			63R:1S	1			2626.8	< 0.00001			1460.2	< 0.00001
			1R:63S	1			4828.2	< 0.00001			6810.6	< 0.00001

* Observed segregation ratio not significantly different from the tested ratio at $P \leq 0.05$.

Table 4.3. Segregation ratios for resistance to clubroot in an ECD 02 × CR 1054 derived F_2 population under greenhouse conditions.

Popl/ Fam	Pathotype	Total No. of F_2 (Score=No. plants)	Tested ratio	DF	Observed (disease score)[1]		Test of statistics [1]		Observed (disease score)[2]		Test of statistics [2]	
					R (0)	S (1+2+3)	χ^2	Prob	R (0 +1)	S (2+3)	χ^2	Prob
Popl#3	3H	109	3R:1S	1	41	68	81.3	< 0.00001	73	36	3.7	**0.0528***
Pooled		(Score 0 = 41)	9R:7S	1			15.4	0.00009			5.1	0.0241
		(Score 1 = 32)	5R:11S	1			2.1	**0.1473***			64.7	< 0.00001
		(Score 2 = 14)	11R:5S	1			49.2	< 0.00001			0.2	**0.6892***
		(Score 3 = 22)	15R:1S	1			586.2	< 0.00001			133.4	< 0.00001
			1R:15S	1			183.0	< 0.00001			685.9	< 0.00001
			63R:1S	1			2621.7	< 0.00001			701.6	< 0.00001
			1R:63S	1			920.1	< 0.00001			3030.5	< 0.00001
Popl#3	5X	144	3R:1S	1	3	141	408.3	< 0.00001	9	135	363.0	< 0.00001
Fam 1		(Score 0 = 3)	9R:7S	1			171.7	< 0.00001			146.3	< 0.00001
		(Score 1 = 6)	5R:11S	1			57.0	< 0.00001			41.9	< 0.00001
		(Score 2 = 10)	11R:5S	1			297.9	< 0.00001			261.8	< 0.00001
		(Score 3 = 125)	15R:1S	1			2065.1	< 0.00001			1881.6	< 0.00001
			1R:15S	1			4.3	0.0381			0.0000	**1.0000***
			63R:1S	1			8692.1	< 0.00001			7956.6	< 0.00001
			1R:63S	1			0.3	**0.5839***			20.4	< 0.00001
Popl#3	5X	28	3R:1S	1	2	26	68.8	< 0.00001	9	19	27.4	< 0.00001
Fam 2		(Score 0 = 2)	9R:7S	1			27.4	< 0.00001			6.6	0.0101
		(Score 1 = 7)	5R:11S	1			7.6	0.0058			0.01	**0.9203***
		(Score 2 = 8)	11R:5S	1			49.5	< 0.00001			17.5	0.00003
		(Score 3 = 11)	15R:1S	1			358.4	< 0.00001			181.4	< 0.00001
			1R:15S	1			0.04	**0.8415***			32.0	< 0.00001
			63R:1S	1			1517.3	< 0.00001			800.1	< 0.00001
			1R:63S	1			5.6	0.0180			170.0	< 0.00001
Popl#3	5G	73	3R:1S	1	38	35	20.5	< 0.00001	49	24	2.4	**0.1198***
Fam 2		(Score 0 = 38)	9R:7S	1			0.5	**0.4795***			3.5	**0.0610***
		(Score 1 =11)	5R:11S	1			14.7	0.0001			43.7	< 0.00001
		(Score 2 = 9)	11R:5S	1			9.5	0.0021			0.09	**0.7642***
		(Score 3 = 15)	15R:1S	1			216.6	< 0.00001			88.3	< 0.00001
			1R:15S	1			261.4	< 0.00001			461.7	< 0.00001
			63R:1S	1			1021.1	< 0.00001			465.4	< 0.00001
			1R:63S	1			1209.2	< 0.00001			2039.0	< 0.00001

* Observed segregation ratio not significantly different from the tested ratio at $P \leq 0.05$

Table 4.4. Polymorphic markers used for linkage map construction and QTL analysis.

Marker	Chrom	Flanked/associated *CR* gene	Forward	Reverse	Reference
GC2360	A03	*CRa*	CAGCACCAGCATAACCAGCTACAGTC	AGAACTTTGCAAGTGGCTCAGATAAT	Matsumoto et al. (2012)
GC2920	A03	*CRa*	CAAAGAACTGCCTGTTGTAAGTAAA	TGTTCAACAAGTTCCCATCTCCAT	Matsumoto et al. (2012)
JY14	A03	*CRa*	GCGTGTTTGATGACTTTCCCT	GGTGGTGGAAACCCTAGGAA	This study
JY44	A03	*CRa*	AGACTTTGCAAGACCTCAACA	CTGAAGAGGAACAGGGTCAT	This study
CRaJY	A03	*CRa*	GTTGGAGACGGAGGTGAAGA	GCATCCCGTGAGATTCAGTT	This study
TCR05	A03	*CRb*	AGAATCATGACCGGGGAAAT	GCAGCTAAGTCATCGACCAA	Piao et al. (2004)
TCR09	A03	*CRb*	GCAGCAACCGATAATATAAGGA	AACCAGAAGAAGAAAAACAAAAA	Piao et al. (2004)
TCR17	A03	*CRb*	GCACATCACTTTGAGGACGA	TTTCCGTTGTCCTTTGTGAA	Piao et al. (2004)
TCR30	A03	*CRb*	CGTGGATCTCGTCTTCAGGT	GGAACAGTATACTTCCCGGTGT	Zhang et al. (2014)
TCR74	A03	*CRb*	ATGGATGATGGATGGATAGAGTG	TTGAACCATAGGAGGGATAGTTG	Zhang et al. (2014)
TCR79	A03	*CRb*	TGACGTTCAATCAAAGCCTGA	TTTAGCAATCAAATGCAAATTCAA	Zhang et al. (2014)
KBrH129J08Rc	A03	CRb^{Kato}	ATGAGATTGAAGAGGGAAACACAA	GTTTCCAATGGTGAAACCAATCCTA	Kato et al. (2012)
KBrH129J18R	A03	CRb^{Kato}	AGAGCAGAGTGAAACCAGAACT	GTTTCAGTTCAGTCAGGTTTTTGCAG	Kato et al. (2012)
KBrB091M11R	A03	CRb^{Kato}	ACTTAAAGCACGAGAATGCAAA	GTTTGGTGTCGAAGCTATGTGTG	Kato et al. (2012)
KB69N05	A03	CRb^{Kato}	ATCACAACCAAAATGGAATGAC	GTTTCTCAAGCACCGAGACTCATAA	Kato et al. (2013)
KB59N06	A03	CRb^{Kato}	ATGAAATTGCAACTCTCAAAATG	GTTTAGGCTTTCTCCATCAACCACTA	Kato et al. (2013)
KB59N03	A03	CRb^{Kato}	AGGTAAATCCTCAAAAAGCCAT	GTTTGGCGAAATTCAGTTGACA	Kato et al. (2013)
B4732	A03	CRb^{Kato}	ATCTGATGTACCTTTGTGCTGG	GTTTGTCAATCATTCAAGCTAAGTGG	Kato et al. (2013)
B0903	A03	CRb^{Kato}	ACTTCCTCTGCTTTTCTCAGGT	GTTTGAAACTCTTCTCCCCCTTC	Kato et al. (2013)
BGA06	A03	CRb^{Kato}	AGAAATAGCAAAGCTCAAACGG	GTTTCCAGAAAAGAGATGCAGACAA	Kato et al. (2013)
BGB41	A03	CRb^{Kato}	ATCGCATAAACTAATAAAAATCAAAA	GTTTGACCCACATGATTAACAA	Kato et al. (2013)
KB29N19	A03	CRb^{Kato}	ATGAGATCGTCAGCCATTTCTC	GTTTCCAGTCCGGTTTTTATTACCTT	Kato et al. (2013)
KB29N16	A03	CRb^{Kato}	AGACTCGACAAGGTATCGATCT	GTTTGACGCCATTATGACACAACT	Kato et al. (2013)
KB29N11	A03	CRb^{Kato}	ACTCTCCACCAACACTTCCTAA	GTTTGAAGCTATCTTAGACCACC	Kato et al. (2013)
KB91N13	A03	CRb^{Kato}	AGACGGAGACTTTGAGATCTGG	GTTTCGAGTACTTCCAGAAACACG	Kato et al. (2013)
Ol11-G11	A03	Crr3	GTTGCGGCGAAACAGAGAAG	GAGTAGGCGATCAAACCGAG	Fredua-Agyeman and Rahman (2016)
BrSTS-020	A03	*Crr3*	CTTCAGAACATCAGAAAGGGTCTT	TTGTTAATCTTGGTTGGGATGTTA	Fredua-Agyeman and Rahman (2016)
BRMS-050	A03	*CRb*	AACTTTGCTTCCACTGATTTTT	TTGCTTAACGCTAAATCCATAT	Fredua-Agyeman and Rahman (2016)
BnGMS344	A03	*CRb*	TGGGAAGAATCTCGTTAGAA	TCTCCTCTTCGGTTACGATA	Fredua-Agyeman and Rahman (2016)

Na10-B01	A03	*CRk*	CAAGTGTCTGCTAGGTGGGG	TCGATCGAAGAAACCAGACC	Fredua-Agyeman and Rahman (2016)
Ol11-B05	A03	*CRk*	TCGCGACGTTGTTTTGTTC	ACCATCTTCCTCGACCCTG	Fredua-Agyeman and Rahman (2016)
Na14-E02	A03	*CRk*	ACTGGCTACATGAGTTTCAGTG	GAGGGAAGACAACTGGTCTCA	Fredua-Agyeman and Rahman (2016)
BRMS-088	A08	*Crr1*	TATCGGTACTGATTCGCTCTTCAAC	ATCGGTTGTTATTTGAGAGCAGATT	Suwabe et al. (2003)
A08-5021	A08	*Crr1*	CAGATGAGACAACACAGGAAACA	ACTCAATACGTTTTTCGCGG	Hobson and Rahman (2016)

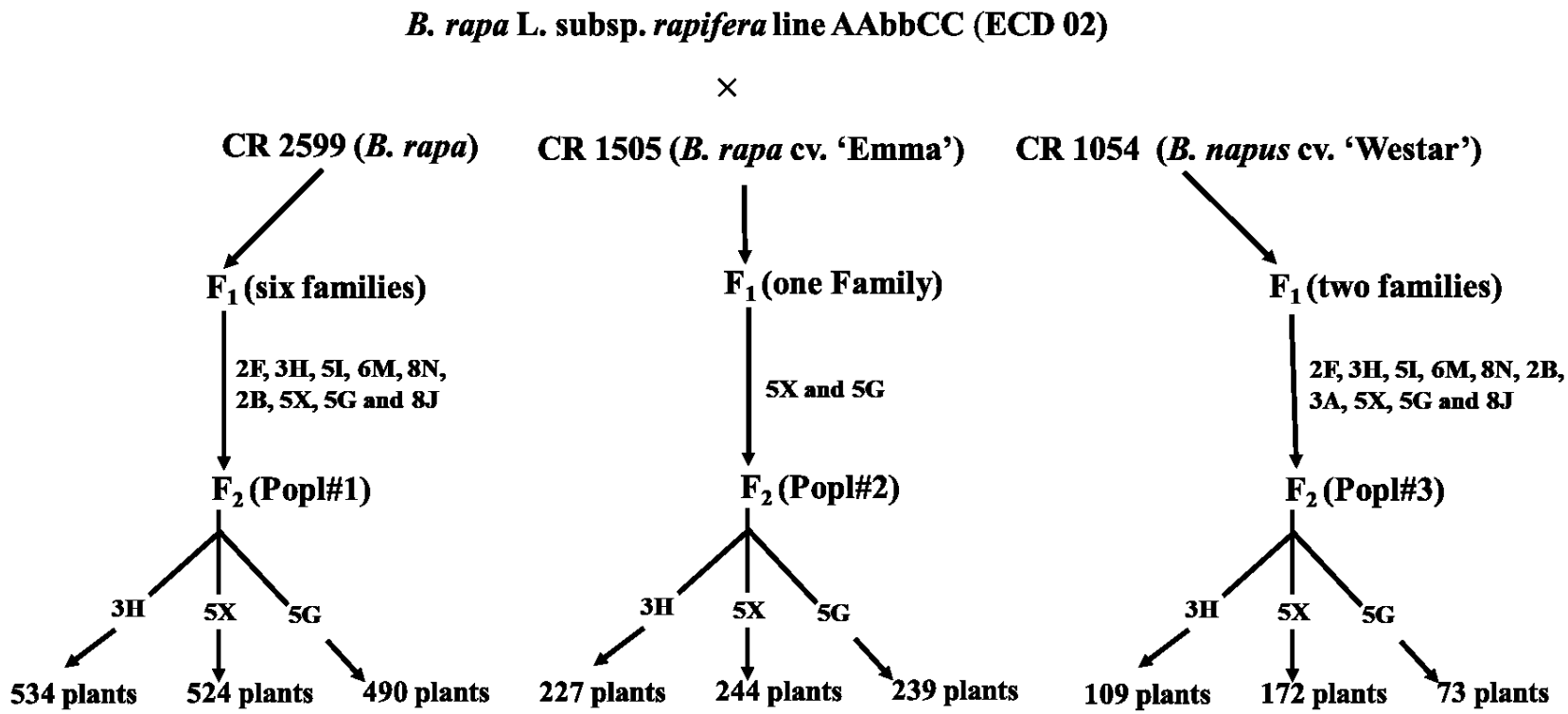

Figure 4.1. Development of an F_2 mapping population for the study of the inheritance of clubroot resistance introgressed from the *Brassica rapa* genotype ECD 02.

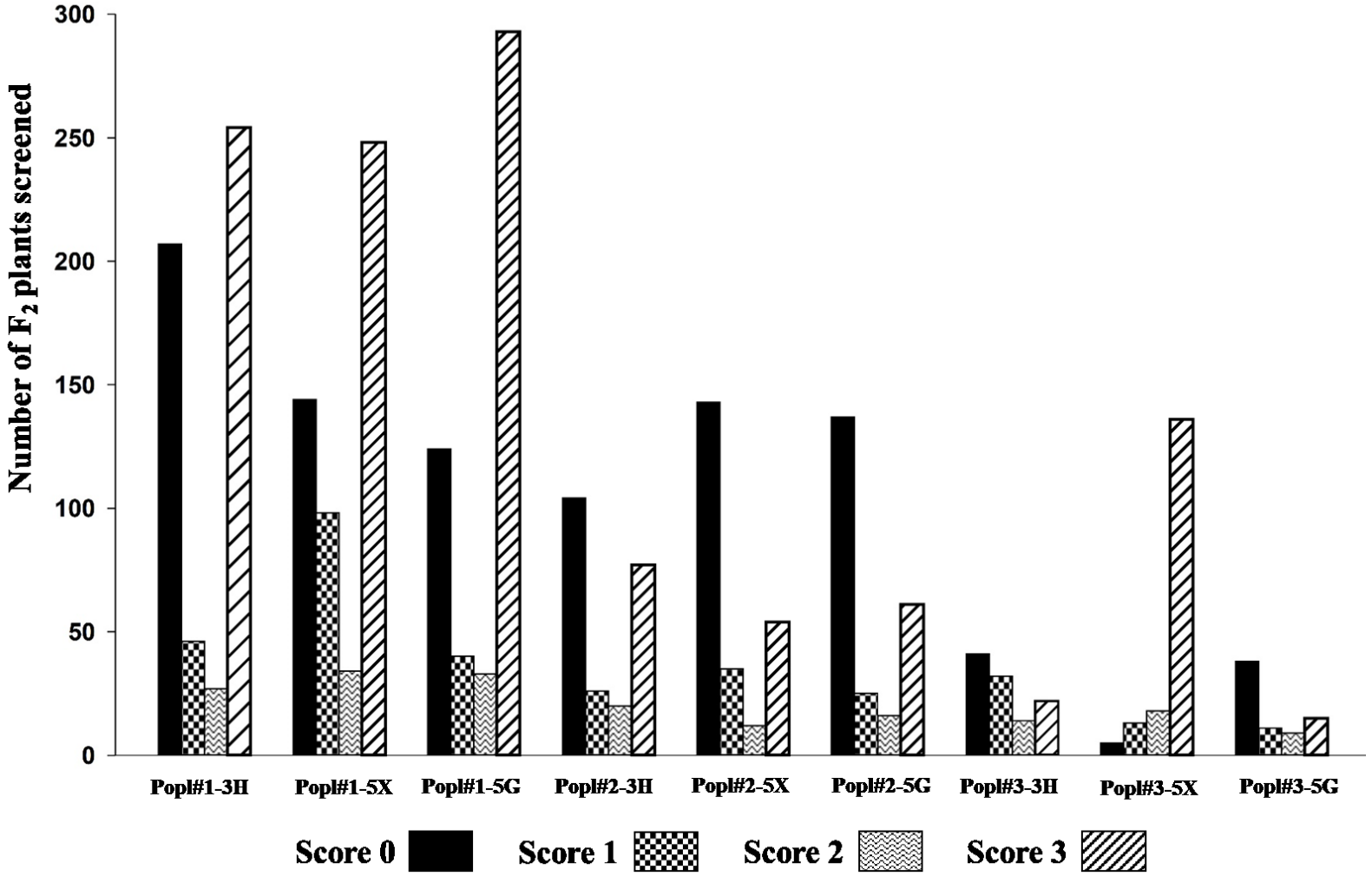

Figure 4.2. Frequency distribution of disease scores obtained in three F_2 populations (Popl#1, Popl#2 and Popl#3) derived from the *Brassica rapa* genotype ECD 02 in response to inoculation with *Plasmodiophora brassicae* pathotypes 3H, 5X and 5G. Scores: 0 = no galls; 1 = a few small galls on < 1/3 of the roots; 2 = medium galls on 1/3-2/3 of the roots, and 3 = large galls on > 2/3 of the roots.

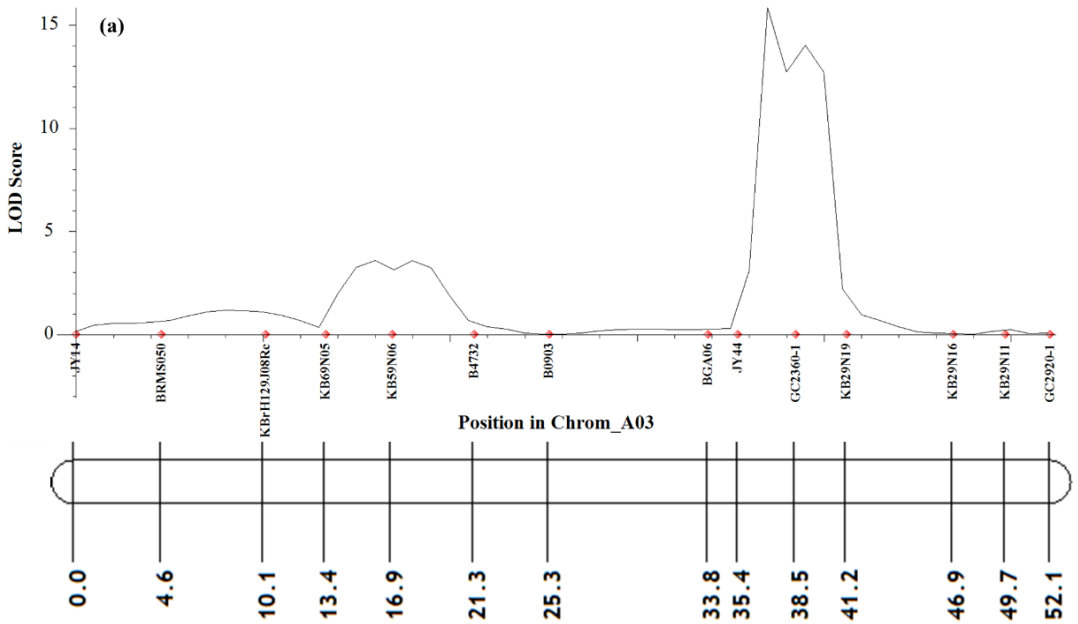

Figure 4.3. QTL likelihood profile and partial linkage map of the A03 chromosome of *Brassica napus* showing resistance to *P. brassicae* pathotype 5X in Popl#1. The LOD scores are indicated on the y-axis while the marker names and genetic distances (in cM) are indicated on the x-axis. The first QTL bordered by markers KB69N05 and B4732 is located between 24274312 to 24348056 nt, while the second QTL bordered by markers BGA06 and KB29N19 is located between 24426905 to 24637310 nt on the corresponding physical map of *B. napus.*

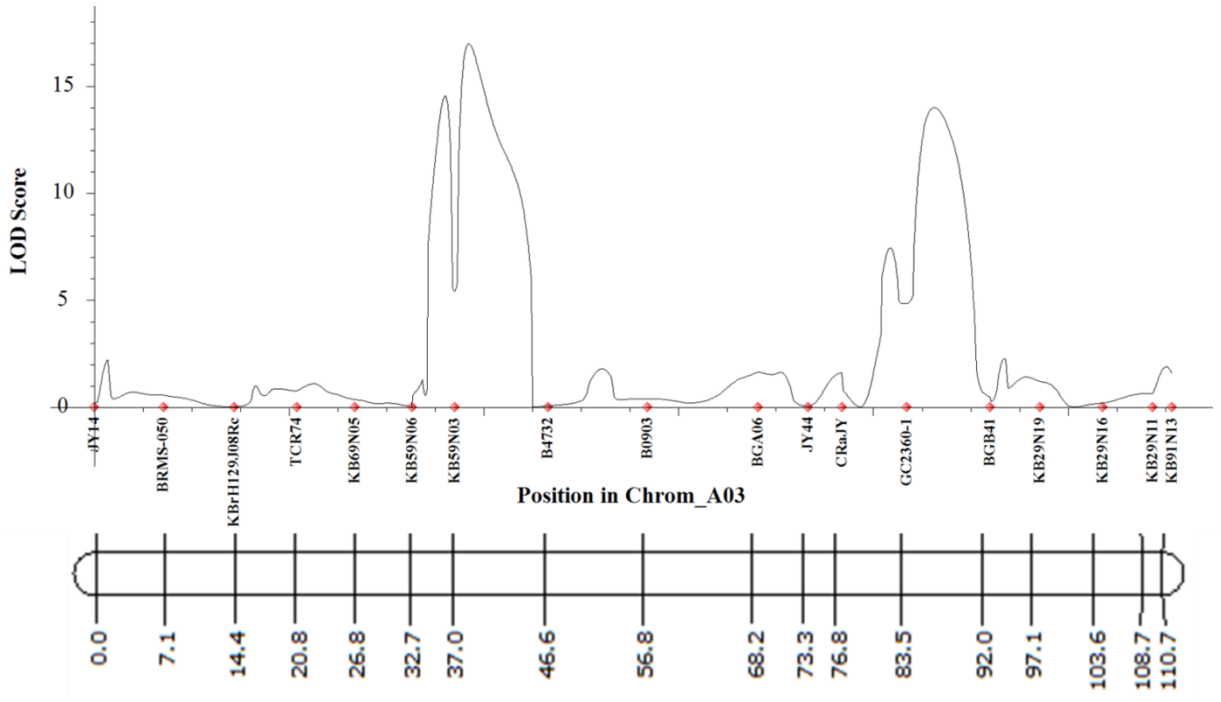

Figure 4.4. QTL likelihood profile and partial linkage map of the A03 chromosome of *Brassica napus* showing resistance to *P. brassicae* pathotype 5G in Popl#1. The LOD scores are indicated on the y-axis while the marker names and the genetic distances (in cM) are indicated on the x-axis. The first QTL bordered by markers KB59N06 and B4732 is located between 24262454 to 24348056 nt, while the second QTL bordered by markers CRaJY and BGB41 is located between 24557499 to 24579679 nt on the corresponding physical map of *B. napus*.

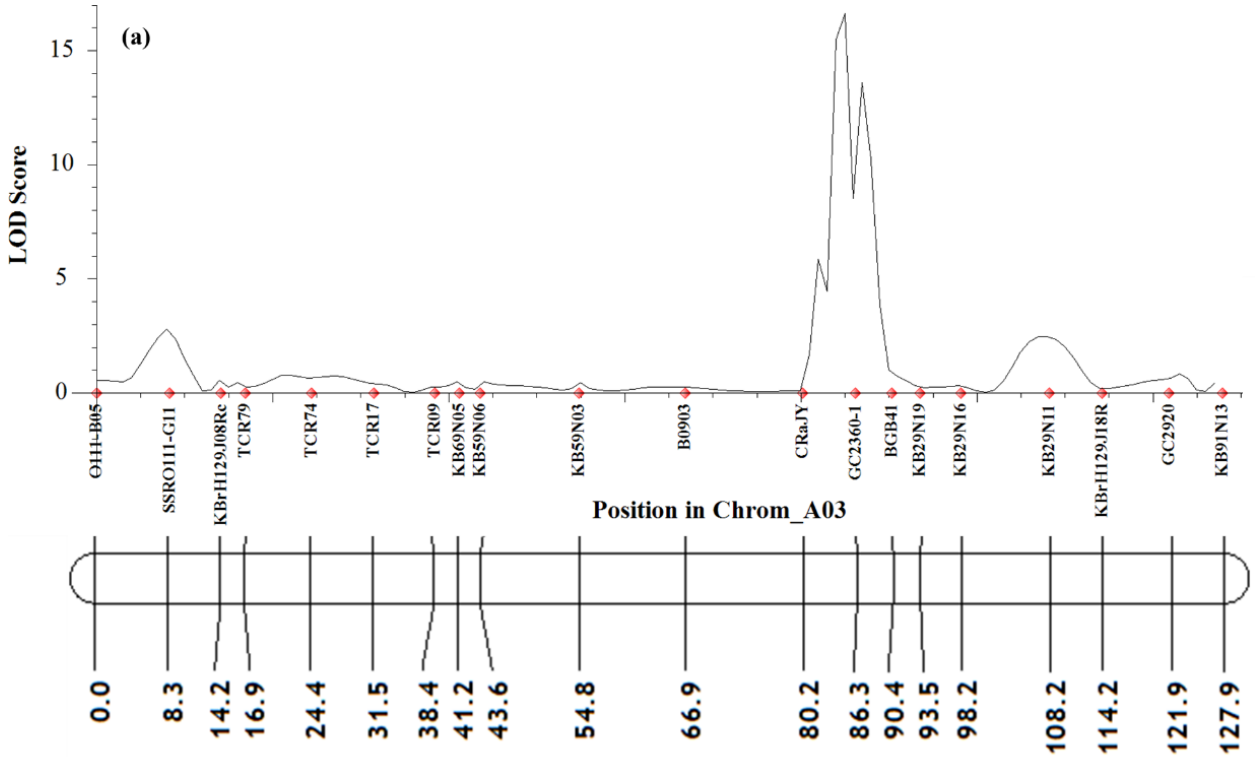

Figure 4.5. QTL likelihood profile and partial linkage map of the A03 chromosome of *Brassica napus* showing resistance to *Plasmodiophora brassicae* pathotype 5X in Popl#2. The LOD scores are indicated on the y-axis while the marker names and genetic distances (in cM) are indicated on the x-axis. The QTL bordered by markers CRaJY and KB29N19 is located between 24557499 to 24637310 nt on the corresponding physical map of *B. napus.*

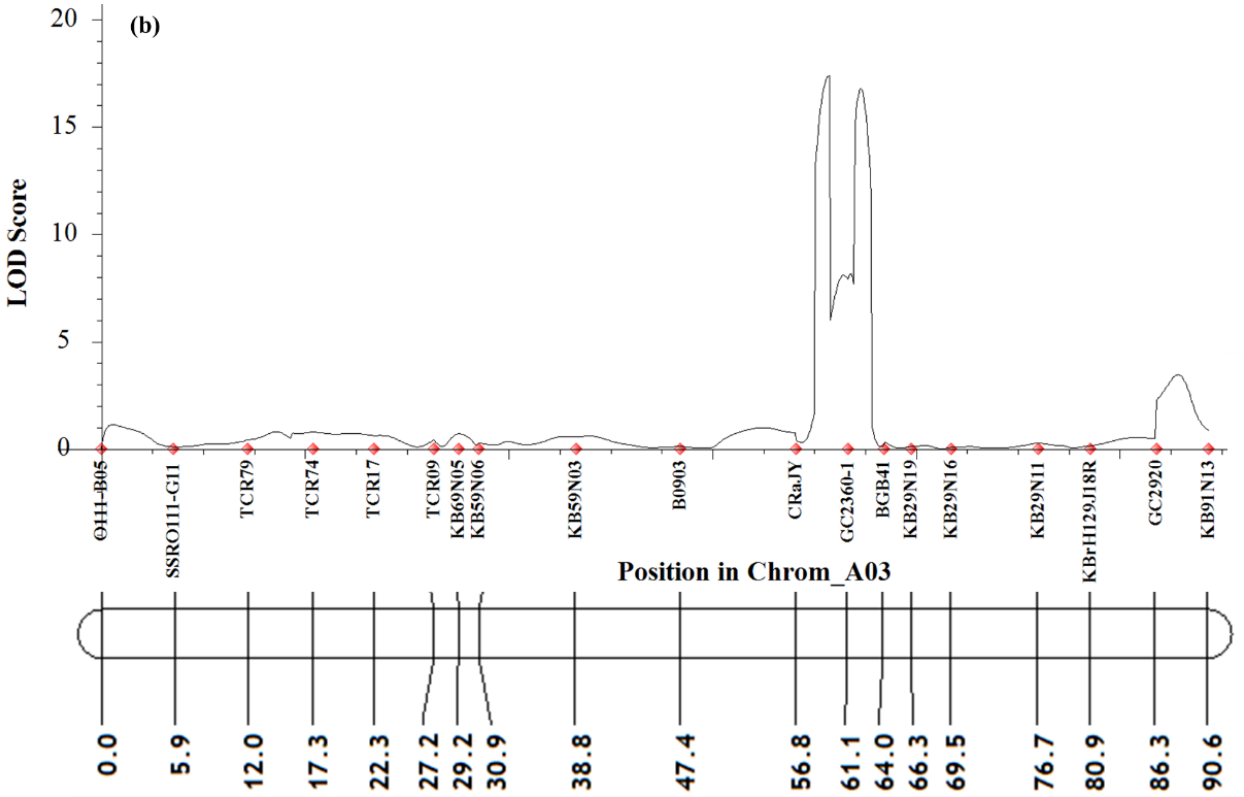

Figure 4.6. QTL likelihood profile and partial linkage map of the A03 chromosome of *Brassica napus* showing resistance to *P. brassicae* pathotype 5G in Popl#2. The LOD scores are indicated on the y-axis while the marker names and the genetic distances (in cM) are indicated on the x-axis. The QTL bordered by markers CRaJY and BGB41 is located between 24557499 to 24579679 nt on the corresponding physical map of *B. napus*.

Chapter 5: General Conclusions

5.1 Primary Infection and Clubroot Development

Primary infection by *Plasmodiophora brassicae* plays an important role in clubroot development, but study of its specific role in pathogenesis is limited. In Chapter 2, I tested the hypothesis that low concentrations of resting spores of virulent isolates of *P. brassicae* can infect the roots of *Brassica napus*, suppressing resistance of the host to avirulent isolates. Clubroot severity was significantly higher in all experimental treatments (low virulent + high avirulent) than in the negative control (high avirulent), but was lower in the experimental treatments than in the positive controls (high virulent, and low virulent + high virulent). Low concentrations of virulent isolates alone caused moderate clubroot. Disease severity was well correlated with *P. brassicae* biomass in canola as determined by quantitative PCR analysis 28-35 days after inoculation. The results of this study suggested that prior exposure of a host to low concentrations of virulent inoculum may compromise resistance and make plants more susceptible to subsequent exposure to high concentrations of avirulent inoculum. More research is needed, however, to understand the mechanisms involved in this effect.

5.2 Study of Genes Differentially Expressed during Pathogenesis

In Chapter 3, I aimed to improve knowledge of the genes expressed by *P. brassicae* during compatible and incompatible interactions with *Brassica napus*. Gene expression by pathotypes 5 and 5X of *P. brassicae* following inoculation of the *B. napus* genotypes '45H29' (susceptible to pathotype 5, resistant to 5X) and 'Westar' (susceptible to both pathotypes) was compared. Pre-screening of 205 genes (coding for proteins with a high cysteine content and/or the presence of an N-terminal RXLR motif) identified 16 genes differentially expressed by pathotypes 5 and 5X on '45H29' 14 days after inoculation (dai). The expression of these genes was further

characterized over a longer time course (7, 14 and 21 dai) in '45H29' and 'Westar'. Twelve of the genes had similar transcriptional patterns in the pathotype 5X/'45H29' and pathotype 5/'Westar' treatments, both of which represented compatible interactions. The similar expression profiles of these genes by two different pathotypes in two different hosts suggests that they may play a role in conferring susceptibility in the host, and might serve as pathogen effectors. While additional research is required to test this hypothesis, it was interesting to find that several of the genes matched entries for effectors and other disease-related proteins in the GenBank database. Further study of these genes at the genomic and proteomic levels may help to determine their role, if any, in clubroot pathogenesis.

5.3 Inheritance of Clubroot Resistance Genes from ECD 02

The European Clubroot Differential (ECD) 02, a genotype of *Brassica rapa*, has proven to be a reliable source of clubroot resistance worldwide. Its potential, however, has not been much explored in the context of the current outbreak of clubroot on *B. napus* in western Canada. In Chapter 4 of this thesis, I crossed ECD 02 with three susceptible accessions including two *B. rapa* (CR 2599 and CR 1505) and one *B. napus* (CR 1054), producing 6, 1 and 2 F_1 families, respectively. After selfing of the F_1 families, 1598, 710 and 354 F_2 individuals derived from the clubroot resistant F_1 families of Popl#1, Popl#2 and Popl#3, respectively, were evaluated for resistance to *P. brassicae* pathotypes 3H, 5X, and 5G. The segregation of clubroot resistance in the F_2 populations largely deviated from the expected Mendelian segregation ratios of 3:1, 9:3:3:1 and 63:1 for resistance controlled by a single, two or three dominant genes, respectively, suggesting additive effects or recessive epistasis. Preliminary molecular marker analysis suggested that one dominant gene and possibly one QTL controlled clubroot resistance introgressed from ECD 02. The presence of at least the two dominant genes on the A03 and the

A08 chromosomes of ECD 02 interacting in a non-allelic manner may help to explain the strong and highly stable qualitative resistance to many pathotypes of *P. brassicae* that has been observed in this host.

5.4 Final Remarks

Clubroot is an important disease of canola and other Brassicas. While genetic resistance is one of the most effective tools to manage *P. brassicae*, in western Canada we have already seen an erosion or loss of resistance that has put this tool at risk. If the long-term management of clubroot is to be achieved, a deeper understanding of the *B. napus*/*P. brassicae* interaction will be required. Such understanding will contribute to the knowledge-based development of clubroot management strategies. Additional proteomic and biochemical experiments will be important in further elucidating clubroot pathogenesis, and the advent of high throughput genomics techniques is already facilititating such lines of research. Additional molecular markers will also need to be identified and anchored on clubroot resistance maps, improving maker-assisted selection for resistance to the many pathotypes of *P. brassicae*. Studies on multiple fronts of the clubroot pathosystem will help to mitigate the impact of this disease.

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Appendix

1. Supplementary Tables from Chapter 2

Table S2.1. The relative quantities (RQs) of *P. brassicae* biomass to canola as determined by qPCR.

Pathotype	Treatment	1 dai	3 dai	5 dai	7 dai	9 dai	11 dai	14 dai	17 dai	21 dai	28 dai	35 dai
2*/2	ET_45H29	1.4 ± 0.8	1.5 ± 0.6	0.1 ± 0.0	2.0 ± 1.1	3.0 ± 0.5	9.2 ± 1.3	3.6 ± 2.6	1.2 ± 0.5	3.0 ± 0.4	34.0 ± 12.3	98.1 ± 66.6
	NC1_45H29	0.0 ± 0.0	0.8 ± 0.4	0.7 ± 0.3	37.0 ± 21.2	1.9 ± 0.4	1.7 ± 1.3	3.2 ± 1.8	0.8 ± 0.3	0.5 ± 0.1	0.9 ± 0.1	0.7 ± 0.6
	NC2_45H29	1.4 ± 0.8	0.2 ± 0.1	0.2 ± 0.1	1.1 ± 0.5	0.5 ± 0.5	0.9 ± 0.6	0.3 ± 0.2	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.4 ± 0.2
	PC1_45H29	1.4 ± 0.8	4.7 ± 2.0	0.5 ± 0.2	5.4 ± 1.1	2.1 ± 0.3	4.7 ± 2.0	9.0 ± 3.7	7.9 ± 4.2	36.5 ± 27.9	35.9 ± 9.8	177.7 ± 76.9
	PC2_45H29	1.2 ± 0.4	1.6 ± 0.5	26.1 ± 3.6	21.7 ± 5.8	9.0 ± 4.7	3.8 ± 1.6	27.6 ± 4.2	49.7 ± 13.7	45.1 ± 16.0	411.8 ± 96.8	580.3 ± 88.0
3*/3	ET_45H29	0.6 ± 0.3	5.5 ± 0.2	1.3 ± 0.2	5.3 ± 2.6	4.2 ± 0.7	8.7 ± 6.0	2.6 ± 2.4	4.1 ± 3.5	6.8 ± 2.4	9.2 ± 3.1	82.2 ± 78.1
	NC1_45H29	0.0 ± 0.0	0.5 ± 0.0	1.0 ± 0.6	2.3 ± 0.4	0.3 ± 0.1	20.2 ± 10.1	3.6 ± 1.8	3.0 ± 1.5	0.4 ± 0.2	0.9 ± 0.3	0.1 ± 0.0
	NC2_45H29	0.6 ± 0.3	0.2 ± 0.1	0.1 ± 0.0	1.5 ± 0.9	0.1 ± 0.0	2.1 ± 0.5	1.0 ± 0.7	0.1 ± 0.0	0.4 ± 0.2	0.0 ± 0.0	0.3 ± 0.0
	PC1_45H29	0.6 ± 0.3	1.9 ± 1.0	1.7 ± 1.1	5.0 ± 1.3	3.2 ± 0.3	8.2 ± 4.9	20.2 ± 9.6	16.7 ± 2.8	25.0 ± 21.3	133.3 ± 81.7	236.2 ± 178.0
	PC2_45H29	3.0 ± 2.6	3.9 ± 1.9	3.9 ± 1.5	13.2 ± 10.0	9.3 ± 4.4	7.6 ± 3.4	26.3 ± 12.5	53.6 ± 38.6	167.0 ± 79.9	249.5 ± 34.2	798.5 ± 119.2
5*/5	ET_45H29	1.0 ± 0.5	3.3 ± 1.5	0.3 ± 0.1	4.1 ± 3.1	3.5 ± 1.9	16.7 ± 10.4	9.9 ± 6.4	2.3 ± 1.1	3.7 ± 1.4	4.3 ± 2.1	23.2 ± 21.0
	NC1_45H29	1.1 ± 1.1	7.8 ± 0.2	0.6 ± 0.3	0.2 ± 0.1	6.8 ± 3.7	4.6 ± 1.8	5.1 ± 2.2	1.8 ± 0.8	4.5 ± 2.7	1.3 ± 0.5	0.7 ± 0.0
	NC2_45H29	1.0 ± 0.5	0.1 ± 0.1	0.1 ± 0.0	4.0 ± 2.9	0.0 ± 0.0	1.3 ± 1.1	0.2 ± 0.1	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.3 ± 0.1
	PC1_45H29	1.0 ± 0.5	0.4 ± 0.2	0.3 ± 0.1	2.2 ± 1.4	0.6 ± 0.0	2.1 ± 1.1	1.0 ± 0.4	0.8 ± 0.3	1.3 ± 0.7	42.8 ± 9.8	160.8 ± 114.8
	PC2_45H29	0.4 ± 0.1	0.1 ± 0.0	1.0 ± 0.5	7.7 ± 5.0	4.5 ± 1.1	21.5 ± 11.3	8.4 ± 7.7	44.0 ± 22.2	93.7 ± 17.1	216.5 ± 72.8	284.7 ± 49.4
2*/2	ET_L135C	0.5 ± 0.3	6.1 ± 1.6	0.1 ± 0.0	14.7 ± 7.5	13.0 ± 8.4	10.9 ± 0.5	6.2 ± 1.7	2.0 ± 1.1	5.4 ± 1.5	7.9 ± 0.9	105.3 ± 50.0
	NC1_L135C	4.0 ± 0.6	0.3 ± 0.1	2.1 ± 0.1	31.2 ± 15.3	3.6 ± 2.5	12.9 ± 9.3	4.3 ± 3.2	3.6 ± 1.8	1.0 ± 0.6	1.4 ± 0.9	1.0 ± 0.3
	NC2_L135C	0.5 ± 0.3	0.4 ± 0.0	0.2 ± 0.0	1.7 ± 0.8	0.0 ± 0.0	2.0 ± 1.1	1.0 ± 0.7	0.0 ± 0.0	1.3 ± 1.3	0.0 ± 0.0	0.5 ± 0.2
	PC1_L135C	0.5 ± 0.3	4.1 ± 2.0	1.1 ± 0.5	3.5 ± 1.1	0.5 ± 0.3	4.9 ± 2.7	18.9 ± 3.8	10.9 ± 9.5	10.2 ± 4.8	49.7 ± 27.7	162.2 ± 23.5
	PC2_L135C	0.6 ± 0.4	0.9 ± 0.6	1.7 ± 0.9	9.0 ± 4.0	11.2 ± 1.8	22.7 ± 2.7	10.8 ± 8.3	22.6 ± 11.1	168.9 ± 69.8	208.1 ± 22.9	563.5 ± 290.3
3*/3	ET_L135C	0.3 ± 0.2	6.8 ± 3.5	1.9 ± 1.0	26.5 ± 5.2	12.2 ± 5.8	13.2 ± 8.3	11.4 ± 1.8	15.6 ± 9.6	12.9 ± 5.3	16.0 ± 4.6	56.0 ± 52.1
	NC1_L135C	4.0 ± 1.5	1.1 ± 0.3	1.1 ± 1.0	1.9 ± 0.3	5.2 ± 3.3	10.9 ± 0.1	5.5 ± 2.6	6.8 ± 3.0	0.8 ± 0.1	0.6 ± 0.3	0.1 ± 0.0
	NC2_L135C	0.3 ± 0.2	0.5 ± 0.0	0.1 ± 0.0	5.5 ± 2.4	0.1 ± 0.0	2.1 ± 0.8	1.5 ± 0.8	0.1 ± 0.1	0.1 ± 0.0	1.1 ± 1.1	0.3 ± 0.0
	PC1_L135C	0.3 ± 0.2	2.5 ± 0.7	0.3 ± 0.1	6.3 ± 3.5	0.1 ± 0.0	18.5 ± 9.5	15.1 ± 3.4	10.9 ± 5.5	47.0 ± 37.6	121.3 ± 52.7	121.6 ± 51.1
	PC2_L135C	2.9 ± 1.3	2.6 ± 2.1	3.2 ± 2.8	10.7 ± 8.3	6.6 ± 5.3	6.1 ± 3.1	5.2 ± 2.6	30.9 ± 4.3	53.6 ± 26.2	478.1 ± 127.0	492.6 ± 174.6
5*/5	ET_L135C	0.5 ± 0.2	7.9 ± 2.9	0.7 ± 0.5	20.9 ± 0.3	0.3 ± 0.2	10.4 ± 2.2	16.4 ± 2.6	7.7 ± 4.2	11.2 ± 5.3	11.1 ± 3.5	23.2 ± 21.5

	NC1_L135C	4.1 ± 0.7	11.4 ± 3.6	1.3 ± 0.6	0.9 ± 0.4	28.0 ± 14.4	7.4 ± 3.5	16.3 ± 6.4	12.3 ± 6.1	1.7 ± 0.8	3.2 ± 1.2	2.9 ± 0.8
	NC2_L135C	0.5 ± 0.2	0.3 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.5 ± 0.2	0.3 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	1.2 ± 0.3
	PC1_L135C	0.5 ± 0.2	0.2 ± 0.1	0.4 ± 0.2	2.0 ± 1.0	3.3 ± 2.2	0.3 ± 0.1	5.3 ± 2.6	2.7 ± 0.7	76.5 ± 73.3	102.3 ± 36.8	143.9 ± 129.0
	PC2_L135C	0.6 ± 0.4	0.1 ± 0.0	3.6 ± 3.0	1.4 ± 0.7	1.2 ± 0.6	12.4 ± 12.2	6.3 ± 2.5	41.7 ± 22.2	40.3 ± 4.5	230.5 ± 47.8	273.9 ± 39.1
2*/2	ET_L241C	1.4 ± 0.7	2.5 ± 0.6	0.2 ± 0.1	3.1 ± 2.2	3.9 ± 1.6	8.1 ± 2.2	5.2 ± 2.9	3.5 ± 1.2	4.3 ± 1.0	13.2 ± 6.8	106.8 ± 67.7
	NC1_L241C	1.6 ± 0.3	1.1 ± 0.1	0.7 ± 0.3	10.7 ± 5.2	19.4 ± 12.9	21.0 ± 13.1	14.0 ± 8.7	0.6 ± 0.2	2.0 ± 0.9	1.9 ± 0.2	1.3 ± 0.6
	NC2_L241C	1.4 ± 0.7	0.5 ± 0.3	0.1 ± 0.0	2.6 ± 1.5	0.1 ± 0.0	0.5 ± 0.1	1.0 ± 0.7	0.2 ± 0.1	1.0 ± 0.5	0.0 ± 0.0	2.8 ± 2.3
	PC1_L241C	1.4 ± 0.7	1.7 ± 1.2	1.0 ± 0.4	10.1 ± 2.1	2.7 ± 1.4	15.2 ± 8.2	52.1 ± 45.9	25.6 ± 8.7	46.2 ± 29.6	183.9 ± 33.5	192.5 ± 71.1
	PC2_L241C	1.1 ± 0.3	1.5 ± 0.9	21.4 ± 7.4	11.8 ± 6.3	14.7 ± 7.1	7.5 ± 1.8	41.4 ± 5.0	53.0 ± 8.0	95.0 ± 36.2	357.5 ± 165.2	475.6 ± 69.0
3*/3	ET_L241C	0.5 ± 0.2	4.2 ± 2.6	0.3 ± 0.1	2.4 ± 1.2	4.1 ± 2.0	18.7 ± 1.8	9.6 ± 0.4	2.8 ± 1.9	10.7 ± 3.5	18.4 ± 8.4	6.3 ± 4.1
	NC1_L241C	5.6 ± 2.5	4.6 ± 1.0	0.9 ± 0.7	0.6 ± 0.2	0.0 ± 0.0	9.7 ± 4.3	7.7 ± 3.4	0.2 ± 0.1	2.3 ± 0.1	4.1 ± 2.2	0.8 ± 0.4
	NC2_L241C	0.5 ± 0.2	0.3 ± 0.0	0.1 ± 0.0	3.1 ± 0.3	0.1 ± 0.0	0.7 ± 0.2	1.0 ± 0.6	0.1 ± 0.0	0.1 ± 0.0	3.3 ± 3.2	1.9 ± 1.0
	PC1_L241C	0.5 ± 0.2	2.5 ± 1.1	0.3 ± 0.1	8.3 ± 3.7	1.4 ± 1.0	9.3 ± 3.3	9.1 ± 2.0	13.6 ± 5.9	10.1 ± 2.7	109.5 ± 51.4	218.7 ± 196.5
	PC2_L241C	2.6 ± 1.6	3.7 ± 0.6	23.5 ± 3.8	15.2 ± 5.1	2.6 ± 2.1	20.7 ± 16.6	26.9 ± 8.7	48.4 ± 9.6	159.1 ± 64.7	329.3 ± 139.6	656.5 ± 297.1
5*/5	ET_L241C	1.0 ± 0.5	10.1 ± 3.4	0.4 ± 0.2	7.3 ± 2.6	1.5 ± 1.1	1.2 ± 0.5	8.4 ± 7.2	4.5 ± 2.1	11.5 ± 1.0	83.7 ± 75.6	93.4 ± 8.2
	NC1_L241C	7.5 ± 3.0	8.8 ± 1.4	1.0 ± 0.5	0.3 ± 0.1	0.3 ± 0.2	28.7 ± 5.3	10.3 ± 5.2	2.1 ± 0.6	3.5 ± 2.4	2.2 ± 0.1	1.8 ± 0.3
	NC2_L241C	1.0 ± 0.5	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.3 ± 0.0	0.7 ± 0.4	6.6 ± 5.6	0.3 ± 0.2	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.1
	PC1_L241C	1.0 ± 0.5	0.6 ± 0.1	0.8 ± 0.1	2.7 ± 0.9	1.1 ± 0.3	2.9 ± 2.3	2.8 ± 1.2	1.6 ± 0.6	54.0 ± 49.0	172.5 ± 28.3	305.2 ± 163.4
	PC2_L241C	1.1 ± 0.8	0.5 ± 0.2	14.2 ± 7.2	6.3 ± 2.6	4.5 ± 4.3	21.6 ± 14.5	42.4 ± 20.7	38.9 ± 16.3	196.2 ± 98.7	744.1 ± 300.3	944.3 ± 238.9

2. Supplementary Tables from Chapter 4

Table S4.1. Clubroot assays of F_1 plants of ECD 02* × [CR 2599$^{\alpha}$, CR 1505$^{\beta}$ and CR1054$^{\gamma}$]

F_1 plants of the cross	*P. brassicae* Pathotype[a]	Disease score[b] 0	1	2	3
ECD 02* × CR 2599$^{\alpha}$	2F	2	0	0	0
	3H	2	0	0	0
	5I	0	2	0	0
	6M	3	0	0	0
	8N	3	0	0	0
	2B	1	0	0	0
	5X (L-G1)	4	0	0	0
	5G	4	1	0	0
	8J	2	1	0	0
	All pathotypes	**21**	**4**	**0**	**0**
ECD 02* × CR 1505$^{\beta}$	5X (L-G1)	1	0	0	0
	5G	1	0	0	0
	All pathotypes	**2**	**0**	**0**	**0**
ECD 02* × CR1054$^{\gamma}$	2F	5	1	0	0
	3H	3	1	0	0
	5I	4	2	0	0
	6M	4	0	0	0
	8N	2	3	0	0
	2B	1	1	0	0
	3A	1	0	0	0
	5X (L-G1)	6	1	0	0
	5G	0	1	0	0
	8J	0	1	0	0
	All pathotypes	**26**	**11**	**0**	**0**
ECD 02* × [CR 2599$^{\alpha}$, CR 1505$^{\beta}$, CR1054$^{\gamma}$]	**Overall**	**49**	**15**	**0**	**0**

* The resistant parent ECD 02 is *B. rapa* L. subsp. *rapifera* line AAbbCC.

$^{\alpha, \beta}$ The susceptible parents CR 2599 and CR 1505 (cv. 'Emma') are *B. rapa* accessions.

$^{\gamma}$ The susceptible parents CR 1054 is *B. napus* cv. 'Westar'.

[a] Pathotypes 2F, 3H, 5I, 6M and 8N are single-spore isolates of *Plasmodiophora brassicae* identified prior to the introduction of clubroot resistant canola in Canada, while pathotypes 2B, 3A, 5X, 5G and 8J are field isolates identified after the introduction of clubroot resistance.

[b] Scores: 0 = no galls; 1 = a few small galls on < 1/3 of the roots; 2 = medium galls on 1/3-2/3 of the roots, and 3 = large galls on > 2/3 of the roots. Numbers beneath the scores indicate the number of F_1 plants with each score.

Table S4.2a. Chi-square tests of homogeneity: F_2 popl#1 screened with pathotype 3H (R = disease score 0; S = disease score 1 + 2 + 3).

F_2 family	No. of F_2 plants with disease score				Total	Obs R	Obs S	Exp R	Exp S	((O-E)^2) /E	χ^2	DF	Prob
	0	1	2	3		(0)	(1+2+3)						
1	10	4	2	18	34	10	24	13.2	20.8	0.77	37.20	5	< 0.00001*
2	39	18	2	9	68	39	29	26.4	41.6	6.06			
3	34	4	11	102	151	34	117	58.5	92.5	10.28			
4	65	12	9	90	176	65	111	68.2	107.8	0.15			
5	39	1	0	10	50	39	11	19.4	30.6	19.86			
6	20	7	3	25	55	20	35	21.3	33.7	0.08			

Table S4.2b. Chi-square tests of homogeneity: F_2 popl#1 screened with pathotype 3H (R = disease score 0 +1; S = disease score 2 + 3).

F_2 family	No. of F_2 plants with disease score				Total	Obs R	Obs S	Exp R	Exp S	((O-E)^2) /E	χ^2	DF	Prob
	0	1	2	3		(0 + 1)	(2+3)						
1	10	4	2	18	34	14	20	16.1	17.9	0.28	46.82	5	< 0.00001*
2	39	18	2	9	68	57	11	32.2	35.8	19.06			
3	34	4	11	102	151	38	113	71.5	79.5	15.73			
4	65	12	9	90	176	77	99	83.4	92.6	0.49			
5	39	1	0	10	50	40	10	23.7	26.3	11.23			
6	20	7	3	25	55	27	28	26.1	28.9	0.03			

* Phenotypic data of the different crosses are significantly different at $P \leq 0.05$ and hence the data were not pooled prior to analysis.

Table S4.3a. Chi-square tests of homogeneity: F_2 popl#1 screened with pathotype 5X (R = disease score 0; S = disease score 1 + 2 + 3).

F_2 family	No. of F_2 plants with disease score				Total	Obs R	Obs S	Exp R	Exp S	((O-E)^2) /E	χ^2	DF	Prob
	0	1	2	3		(0)	(1+2+3)						
1	25	19	4	28	76	25	51	20.9	55.1	0.81	30.40	5	< 0.00001*
2	4	10	3	30	47	4	43	12.9	34.1	6.15			
3	59	27	6	50	142	59	83	39.0	103.0	10.23			
4	47	30	13	73	163	47	116	44.8	118.2	0.11			
5	2	7	7	40	56	2	54	15.4	40.6	11.65			
6	7	5	1	27	40	7	33	11.0	29.0	1.45			

Table S4.3b. Chi-square tests of homogeneity: F_2 popl#1 screened with pathotype 5X (R = disease score 0 +1; S = disease score 2 + 3).

F_2 family	No. of F_2 plants with disease score				Total	Obs R	Obs S	Exp R	Exp S	((O-E)^2) /E	χ^2	DF	Prob
	0	1	2	3		(0 + 1)	(2+3)						
1	25	19	4	28	76	44	32	35.1	40.9	2.26	24.65	5	< 0.00016*
2	4	10	3	30	47	14	33	21.7	25.3	2.74			
3	59	27	6	50	142	86	56	65.6	76.4	6.36			
4	47	30	13	73	163	77	86	75.3	87.7	0.04			
5	2	7	7	40	56	9	47	25.9	30.1	10.99			
6	7	5	1	27	40	12	28	18.5	21.5	2.27			

* Phenotypic data of the different crosses are significantly different at $P \leq 0.05$ and hence the data were not pooled prior to analysis.

Table S4.4a. Chi-square tests of homogeneity: F_2 popl#1 screened with pathotype 5G (R = disease score 0; S = disease score 1 + 2 + 3).

F_2 family	No. of F_2 plants with disease score				Total	Obs R	Obs S	Exp R	Exp S	((O-E)^2) /E	χ^2	DF	Prob
	0	1	2	3		(0)	(1+2+3)						
1	8	4	0	13	25	8	17	6.3	18.7	0.44	2.24	5	0.8146
2	8	6	6	27	47	8	39	11.9	35.1	1.27			
3	42	12	8	88	150	42	108	38.0	112.0	0.43			
4	38	11	12	95	156	38	118	39.5	116.5	0.06			
5	12	2	3	33	50	12	38	12.7	37.3	0.03			
6	16	5	4	37	62	16	46	15.7	46.3	0.01			

Table S4.4b. Chi-square tests of homogeneity: F_2 popl#1 screened with pathotype 5G (R = disease score 0 +1; S = disease score 2 + 3).

F_2 family	No. of F_2 plants with disease score				Total	Obs R	Obs S	Exp R	Exp S	((O-E)^2) /E	χ^2	DF	Prob
	0	1	2	3		(0 + 1)	(2+3)						
1	8	4	0	13	25	12	13	8.4	16.6	1.58	2.70	5	0.7458
2	8	6	6	27	47	14	33	15.7	31.3	0.19			
3	42	12	8	88	150	54	96	50.2	99.8	0.29			
4	38	11	12	95	156	49	107	52.2	103.8	0.20			
5	12	2	3	33	50	14	36	16.7	33.3	0.45			
6	16	5	4	37	62	21	41	20.8	41.2	0.00			

* Phenotypic data of the different crosses are not significantly different at $P \leq 0.05$ and hence data were pooled prior to analysis.

Table S4.5a. Chi-square tests of homogeneity: F_2 popl#3 screened with pathotype 3H (R = disease score 0; S = disease score 1 + 2 + 3).

F_2 family	No. of F_2 plants with disease score				Total	Obs R	Obs S	Exp R	Exp S	((O-E)^2) /E	χ^2	DF	Prob
	0	1	2	3		(0)	(1+2+3)						
1	36	20	6	20	82	36	46	30.8	51.2	0.86	3.48	1	0.0625
2	5	12	8	2	27	5	22	10.2	16.8	2.62			

Table S4.5b. Chi-square tests of homogeneity: F_2 popl#3 screened with pathotype 3H (R = disease score 0 + 1; S = disease score 2 + 3).

F_2 family	No. of F_2 plants with disease score				Total	Obs R	Obs S	Exp R	Exp S	((O-E)^2) /E	χ^2	DF	Prob
	0	1	2	3		(0 + 1)	(2+3)						
1	36	20	6	20	82	56	26	54.9	27.1	0.02	0.09	1	0.7692
2	5	12	8	2	27	17	10	18.1	8.9	0.06			

* Phenotypic data of the different crosses are not significantly different at $P \leq 0.05$ and hence data were pooled prior to analysis.

Table S4.6a. Chi-square tests of homogeneity: F_2 popl#3 screened with pathotype 5X (R = disease score 0; S= disease score 1 + 2 + 3).

F_2 family	No. of F_2 plants with disease score				Total	Obs R	Obs S	Exp R	Exp S	((O-E)^2) /E	χ^2	DF	Prob
	0	1	2	3		(0)	(1+2+3)						
1	3	6	10	125	144	3	141	4.2	139.8	0.34	2.06	1	0.1508
2	2	7	8	11	28	2	26	0.8	27.2	1.73			

Table S4.6b. Chi-square tests of homogeneity: F_2 popl#3 screened with pathotype 5X (R = disease score 0 +1; S= disease score 2 + 3).

F_2 family	No. of F_2 plants with disease score				Total	Obs R	Obs S	Exp R	Exp S	((O-E)^2) /E	χ^2	DF	Prob
	0	1	2	3		(0 + 1)	(2+3)						
1	3	6	10	125	144	9	135	15.1	128.9	2.44	15.02	1	< 0.00001*
2	2	7	8	11	28	9	19	2.9	25.1	12.57			

$^{\Phi}$ Chi-square test of homogeneity cannot be pooled because 25-50% of the calculated expected (E) values were < 5.