Agriculture and Agri-Food Canada | Agriculture et Agroalimentaire Canada
Saskatoon Research and Development Centre *Project #20140217*

Developing canola germplasms with diverse mechanisms to enhance the durability of clubroot resistance

(Final Report, April 4, 2018)

Gary Peng and Fengqun Yu

Executive summary

Since the introduction of first resistant canola cultivar in 2009, genetic resistance has been a cornerston in clubroot management in Canada. Canola can be grown again in many infested fields with severe clubroot damage previously in central Alberta. In 2013, however, some fields seeded with a resistant canola cultivar showed substantial levels of clubroot damage. Further investigation showed that the pathogen strains from these fields were virulent to each of the resistant cultivars in the marketplace, indicating a potential breakdown of clubroot resistance by new pathogen strains referred tentatively as pathotype 5x. In previous projects, we had screened a large number brassica asscessions for resistance against the pathotype 3 of *Plasmodiophora brassicae*, the most common strain on the prairies, and identified more than twenty resistant candidates. Some of then were even resistant to all pathotypes (2, 3, 5, 6, 8) found in Canada. Clubroot resistance (CR) genes in some of the candidates have been characterized with markers developed for marker-asssited selection. There seemed to be further variations among different populations of pathotype 5x that showed different virulence toward resistant canola cultivars. It was not clear if any of the CR genes we had identified would be effective against all pathotype 5x populations. The goal of this study was to assess if any of the CR genes identified previously would be of efficacy against pathotype 5x of *P. brassica*, explore molecular and biochemical tools that can be used for studying CR mechanisms, and develop canola germplasm carrying more diverse CR genes for sustainable clubroot resistance. The specific objectives included to: 1) assess canola germplasms carrying different CR genes against newly found *P. brassica* pathotypes capable of overcoming the resistant canola cultivars available in the marketplace; 2) characterize resistance mechanisms associated with specific CR genes or gene combinations to learn more about the critical modes of action by the CR gene(s) using transcriptome, proteome, metabolome and histochemical approaches; 3) incorporate effective and versatile CR genes into elite canola breeding lines singly or by stacking for tech transfer.

The project started with screening of 24 resistant candidates (to pathotype 3) against mixed and single populations of pathotype 5x from Alberta fields where resistant cultivars failed in 2013. Several candidates were resistant to mixed 5x populations. These Candidates included those that carry the CR genes *Rcr1, Rcr2, Rcr3* and *Rcr6* identified previously from *B. rapa* and *B. nigra*. These CR genes showed resistance to all other Pb pathotypes found in Canada earlier

(pathotype 2, 3, 5, 6, 8). *Rcr1*(A03) and *Rcr3* (A08), identified in *B. rapa*, showed differential resistance against 5x; *Rcr1* is generally susceptible while *Rcr3* showed resistance to certain 5x populations, especially to DG-3. *Rcr6* from *B. nigra* (B genome) showed resistance to all *P. brassicae* pathotypes or populations found in Canada, including 5x.

Leading-edge technologies were explored to decipher the mechanisms associated with specific CR genes, including transcriptome (RNA-seq), proteome, metabolome, fluorescent microscopy and synchrotron-based chemometrics. These technologies can be used to identify metabolic or signaling pathways relating to clubroot resistance, potentially differentiating the modes of action among different CR genes and guiding CR-gene deployment judiciously to enhance the resistance durability. RNA-seq had been used to identify the molecular modes of action for the CR gene *Rcr1* before the start of this project, with jasmonic acid/ethylene and callose deposition identified as the key signaling and metabolic pathways in mediating clubroot resistance in *B. rapa*. Proteome and metabolome analysis conducted during the period of this project showed further depth of resistance mechanism of *Rcr1*. A total of 527 differentially accumulated proteins (DAPs) were identified in resistant plants, with 296 being increased and 231 decreased, relative to susceptible samples. 523 of these DAPs were annotated to several biological processes, including signaling, biological regulation and response to stimulus. These results indicate that the perception of Pb and subsequent activation of defense responses is triggered likely via a novel signaling pathway requiring the ubiquitin-26S, a proteasome reported frequently in plant cold tolerance responses. It is interesting that similar pathways are activated when plants respond to clubroot infection and cold stress. This was the first report on proteomics on clubroot resistance; it provides insights into the resistance conferred by *Rcr1* and this work has been published in *Frontiers in Plant Science* (Song et al. 2016).

Metabolome profiling was performed at the McGill University, with 153 differentially accumulated metabolites (DAMs) identified in plants carrying *Rcr1*. These DAMs are in several chemical groups, including phenol, alcohol and fatty acids. The accumulation of jasmonic acids as well as the metabolites involved in its signal transduction was significantly increased. Several flavonoids, including the phytoalexin caulilexin C, were also induced. Many of these flavonoids have shown anti-microbial activities against other pathogens.

Synchrotron-based fourier transform mid Infrared (FTIR) spectroscopy identified clear changes in the cell-wall composition associated with the resistance conferred by *Rcr1*, especially the increase in lignin composition. There were significant differences in the absorption of OH, CH_2 and CH_3 and fingerprint regions (1800-800 cm^{-1}) where cell wall constituents could be assigned and distinguished. The cell-wall components were changed in roots carrying *Rcr1*, including lipids, proteins, lignin, phenolics, cellulose/hemicellulose and carbohydrates. A companion RT-qPCR assay found the higher expression of *BrPAL1* in resistant roots; the up-regulation of this *PAL* gene is indicative of an inducible defence response mediated by *Rcr1* that activates this basal defence gene possibly via the phenylpropanoid pathway.

Further work looked at several canola lines carrying a single CR gene that showed resistance against mixed populations of pathotype 5x by inoculation with two 5x populations (L-G2 and L-G3) separately. These CR genes are ineffective against these highly virulent 5x population. Additional hybrid canola (*B. napus*) lines carrying double CR genes were generated through collaborations with breeding companies and tested against the same virulent populations of 5x. The results showed that crosses between the DH line carrying CRB (A8, as a female) and the line carrying *Rcr1* (A3) or CRM (A3) as a male would result in moderate resistance to both L-G2 and L-G3 populations, while the reciprocal crosses of these lines failed to produce the effect. The genetic basis for this differential resistance of reciprocal crossing is not yet well understood; there seem to be cytoplasmic factors associated with the line carrying CRB that can interact with *Rcr1* or CRM in the nuclear to achieve a moderate level of resistance. It is not clear whether these lines are of real value in providing sufficient resistance against most of the pathotype variants identified in Alberta (Stelkov et al. 2018) and can be reasonably durable when exposed repeatedly to the same *P. brassicae* population. This work is being continued in a separate study also supported by SaskCanola and WGRF.

Microscopic examination showed differential clubroot infection in the roots of two double CR-gene canola lines when compared to single CR-gene or susceptible canola; plasmodia or zoosporangia were observed in epidermal cells of the double and single CR-gene/Westar roots, but the percentage of cells with these pathogen structures was substantially lower in the double CR-gene lines. In cortical tissues of moderately resistant roots, the incidence of plasmodia and zoosporangia was even much lower than in epidermal cells or not observed. These results indicate that these double CR-gene lines, despite a moderate level of resistance, effectively reduce the cortical infection. The minor root swelling symptoms may be caused by limited infection of the epidermal layer, which may not have substantial effect on root development. At 12 to 35 days after inoculation, lignification of parenchyma cell wall appeared to be stronger in roots of double CR-gene lines relative to those of Westar. This is direct evidence of cell well modification involved with clubroot resistance, which supports the transcriptome and FTIR results. The molecular mechanisms of double CR-gene lines are being investigated.

Using Bulked sergeant RNA sequencing, gene-specific SNP markers were developed for the CR genes *Rcr1, Rcr2, Rcr3* and *Rcr6* with differential efficacy against *P. brassicae* pathotypes found in Canada, including 5x. MAS was used to introgress these CR genes into *B. napus* (*Rcr1, Rcr2, Rcr3*), *B. carinata* (Rcr6) and *B. juncea* (*Rcr1* and *Rcr6*) canola lines. These CR genes are now in elite AAFC breeding lines with homozygous BC5 seed ready for tech transfer, except *B.* juncea which has showed extremely low fertility rate during inter-specific crossing. Resynthesizing techniques have been used to cross between *B. rapa* (*Rcr1, Rcr4*) and *B. nigra* (*Rcr6*) to generate new resistant *B. juncea* germplasm. Additionally, *Rcr1* (A3), *Rcr2* (A3) and *Rcr3* (A8) have also been transferred to 8 breeding companies via a non-royalty agreement to make hybrids with multiple CR genes, and some of these hybrids have been in field testing in Alberta since 2016. These new CR canola germplasm included: single CR genes on A2, A3 and A8 of A genome of Brassica, a single CR gene on B genome, and multi-genic germplasms

carrying CR genes on A3 and A8. More recently, the resistant *B. rapa* line T19 was crossed with the *B. oleracea* (C genome) to produce resynthesized *B. napus*. It has been shown that the new *B. napus* lines carry multiple CR genes and are resistant to each of the 5x populations, as well as the common pathotypes found in Canada. These lines are being subjected to DH line production for further investigation. The CR gene *Rcr6* (B genome) also appears promising against all pathotypes or variants found in Canada, but it will be more challenging to incorporate it into *B. napus* (AC genome) without sacrificing the yield and quality of canola varieties substantially.

CR genes on A3 chromosome appeared less effective against pathotype 5x when used alone, but reduced clubroot development by ~80% when combined with a CR gene on A8. The CR gene on A2 (*Rcr4*) appeared highly effective against 5x, but not so much to pathotype 3. Staking of CR genes on A2 or A8 with any of those on A3 may be of value in terms of broadening the resistance against more pathotypes and new crosses will be made to stake these two CR genes. There is also evidence that the double CR-gene lines seem to be durable under repeated exposure to the same population of *P. brassicae*. It is becoming more clear that the clubroot pathogen population is more diverse than what we knew before, and there will likely be further variants to be identified. Therefore, it will be important to better understand the resistance mechanisms with the CR germplasms developed for effective deployment. The work under this project helped equip us with more advanced technologies, including transcriptome, proteome, metabolome and synchrotron-based FTIR spectroscopy for studying resistance modes of action; a comparative study of single and multi CR-gene lines with differential efficacy against the 5x and other pathotype variants will provide more information for CR gene selection and deployment. A RFP process will be used for tech transfer of *Rcr6* to the industrial oil sector (*B. carinata*), and this would be the first B-genome CR gene to be used for clubroot control.

Overall, with a modest budget the project explored the use of proteomics, metabolomics and synchrotron-based FTIR spectroscopy to study mechanisms associated with specific CR genes and successfully developed a range of canola germplasms carrying single/multi CR genes against the new strain of *P. brassicae* pathotypes 5x. Several stable populations carrying a single CR gene have been transferred to seed industry for the production of new resistant canola hybrids. The project has also generated new materials, especially two resynthesized species and two B. napus breeding lines carrying double CR genes, for further studies on resistance mechanisms and durability in new projects. The project is complete on time and on budget. We sincerely thank SaskCanola and Western Grain Research Foundation for providing financial support to this research.

Background

Clubroot disease continues to be a threat to the canola production in western Canada. Cultivar resistance is considered the most effective and practical approach for clubroot management and resistant canola cultivars has been available in Canada since 2009. All these cultivars are based

on a single clubroot resistance (CR) gene and this type of resistance can be vulnerable because the pathogen population in the soil is diverse and the resistance can be eroded when the pathogen changes in virulence.

CR genes have been reported in *B. rapa* (A genome), *B. nigra* (B genome), *B. oleracea* (C genome) and *B. napus* (AC genome). In prior projects, a large number of brassica secessions were screened and many of candidates showed strong resistance against pathotypes 2, 3, 5, 6 and 8 of *P. brassica* found in Canada (Hasan et al. 2012; Peng et al. 2014; Rahman et al. 2014). Several CR genes (*Rcr1, Rcr2, Rcr3* and *Rcr6*) were also mapped from *B. rapa* and *B. nigra* against *P. brassica* pathotype 3, with markers for some of the CR genes identified (Chu et al. 2014, Yu et al. 2016). Molecular markers closely linked to CR genes can be used for marker-assisted selection (MAS) during introgression of CR genes into elite canola germplasm.

In 2013, some fields in Alberta with a resistant canola cultivar showed substantial levels of clubroot damage. Further testing showed that the pathogen strains from fields were virulent to each of the clubroot resistant cultivar in the marketplace, indicating potentially the poor diversity of CR genes used in Canada. Due to the limitation of current host differentials, these new strains were not separable from the pathotype 5 in the William's system (Williams 1966). These strains were therefore called pathotype 5x tentatively. There are different populations in the 5x group causing different reactions of certain CR genes. It was not clear if any of the CR genes identified would be effective against "new" pathotypes (5x) that are eroding the resistance in commercial CR cultivars.

Along with the resistance towards different pathotypes, understanding the mechanism of CR genes will help select more effective CR candidates for resistance breeding. Multi-genic canola varieties with CR genes of different modes of action may be more durable than mono-genic ones, but the information and tools for conducting studies on CR gene functions are generally lacking; hampers effective use of CR genes in resistance breeding. Our current knowledge suggests that clubroot resistance is expressed most strongly during the secondary infection process (Hwang et al., 2012, Deora et al. 2012), but it is unclear what and how the resistance mechanisms affect the penetration of epidermal cells or the movement of secondary plasmodia in cortical tissues.

Aa group of glucosinolates, secondary metabolites found only in the family of Brassicaceae, have been linked to the development of clubroot symptoms (Ludwig-Müller et al. 1997); aliphatic glucosinolates can be defense compounds (Ludwig-Müller 2009) whereas indole glucosinolates, which can act as precursors for auxin biosynthesis, would favor clubroot development (Ludwig- Müller et al. 1999). Our initial work showed higher overall amounts of glucosinolates in a susceptible line but no substantial increase in any of the resistant lines (data not shown). It is not clear whether lacking certain glucosinolates would stimulate clubroot resistance. Other plant metabolites have been considered as anti-microbial agents, including phytoanticipins and phytoalexins, both can be produced in *B. napus* upon clubroot infection (Pedras et al. 2008). Metabolomics may help answer some of the questions.

Transcriptome and proteome analyses are other leading edges to elucidate host-pathogen interactions (Siemens et al. 2006, Cao et al 2008). Transcriptome analysis helps differentiate genetic alterations between resistance and susceptibility (Chu et al. 2014). Proteomes can be the functional output for gene expression linked to certain transcripts, thus providing confirmation for the genetic analyses by identifying specific resistance-related proteins. Proteomic analysis may also identify critical defense responses at post-transcriptional levels, which would not be shown by the genetic analysis alone, thus helping identify roles of certain differentially produced proteins involved in host defense responses. Kaur et al. (2011) found 19 proteins with differential abundances between resistant and susceptible *B. juncea* to white rust (*Albugo candida*),with five of them displaying exclusive accumulation in resistant plants only. We have developed RNA-seq as a tool for transcriptome analysis (Chu et al. 2014), but proteome and metabolome information was not available with any of the CR genes identified, and their potential in characterizing CR genes for resistance mechanisms was unclear.

The goal of this study was to assess if any of the CR genes identified previously would be of efficacy against pathotype 5x of *P. brassica*, explore molecular and biochemical tools that can be used for studying CR mechanisms, and develop canola germplasm carrying more diverse CR genes for sustainable clubroot resistance. The specific objectives included to: 1) assess canola germplasms carrying different CR genes against newly found *P. brassica* pathotypes capable of overcoming the resistant canola cultivars available in the marketplace; 2) characterize resistance mechanisms associated with specific CR genes or gene combinations to learn more about the critical modes of action by the CR gene(s) using transcriptome, proteome, metabolome and histochemical approaches; 3) incorporate effective and versatile CR genes into elite canola breeding lines singly or by stacking for tech transfer.

Research plan and methodology

1. CR genes/lines and pathogen inoculum

Canola lines carrying the CR genes *Rcr1, Rcr2*, *Rcr3* and *Rcr6* (Owned by AAFC) were tested against the new virulent pathotypes. Potentially new CR genes from the *B. nigra* CR 2716, eight *B. rapa*, one *B. napus* (rutabaga), and six *B. oleracea* lines will also be evaluated against the new pathotypes 5x. Trails were carried out at U of A (Dr. Strelkov) using pathotype 5x populations.

Soil and gall samples were collected from the resistant canola cultivar 45H29 grown an a heavily infested field on the AAFC Normandin Reseach Farm, Qubec, as additional inoculum virulent to the resistance cultivar. Most of the resistant cultivars tested there in 2011 and 2012 were highly resistant to clubroot (Peng et al. 2015).

2. Study of clubroot-resistant mechanisms

***Analysis of global transcriptomes*:** RNA from clubroot susceptible (CS) and CR bulks were

extracted and used for RNA sequencing (RNA-seq). The key objective of this step is to identify genes, including non-coding RNA, that display differential transcriptional patterns (including variable splicing patterns) in roots CR and CS plants, which implies their potential association with the resistance or pathogenicity. The RNA samples wereisolated using a Qiagen RNeasy Plant miniprep kit and treated with DNase to remove genomic DNA contamination. The RNA quantity and quality weredetermined on a Nanodrop Spectrometer and Agilent Bioanalyzer 2100 to generate a RNA integrity number (RIN). High quality samples with a RIN >8 wereused for RNA sequencing.

Raw-reads data werealigned and annotated to reference A-genome and B-genome or using the software Short Oligonucleotide Analysis Package (Wang et al., 2012). Gene expression pattern wereexamined according to RPKM (reads per kb per million reads; Mortazavi et al., 2008) and differentially expressed genes (DEGs) identified following the protocol of Audic and Claverie (1997). Identified DEGs weresubjected to a Gene Ontology analysis (http://www.geneontology-.org/) and RT-qPCR for validation. Alternative splicing and identification of novel transcripts wereperformed as described by Wang et al. (2012). Comparisons weremade initially between CR and CS lines and then among CR lines to identify DEGs potentially involved in clubroot resistance. with gene ontological annotation, some metabolites related to DEGs may be implicated and linked to CR modes of action. These metabolites may be used as biochemical markers for differentiating mechanisms of different CR genes/lines.

***Proteomic analysis*:** A shotgun biological mass spectrometry approach wereused for this study with a dual-pressure linear ion trap and a quadrupole-Orbitrap using nano-flow high performance liquid chromatography (HPLC) interfaced via electrospray ionization. Nanoflow HPLC has extremely high sensitivity. Mass spectrometry (MS) data analyses are conducted using Proteome Cluster, the industry-leading, cloud-based platform for shotgun proteomic data analysis. Multiple search algorithms wereused for optimal confirmatory and complementary results to provide maximum sensitivity while preserving specificity. This analysis can be contracted to Bioproximity (Chantilly, VA) with which we have had contracts for proteomic analysis before.

***Metabolic changes associated with clubroot resistance*:** The focus of this step wereon the analysis of differential regulation of metabolism between CR and CS lines, as well as among CR lines, to understand and differentiate key biochemical factors in clubroot resistance. Root samples werefreeze dried and analyzed using LTQ-Orbitrap MS (De Vos, et al. 2012). This analysis will possibly identify metabolites associated with different CR genes, thus supporting the selection of CR genes with different action modes for clubroot resistance. This analysis can be contracted to MetaboloMetrics Inc. (Montral, QC) with which we have had contracts for metabolomic analysis before. These results werecompared with those from the transcriptomic

and proteomic studies to validate and differentiate the functions of CR genes.

Synchrotron-based FTIR spectroscopic analysis: All infrared data were collected on a mid-infrared beamline (Canadian Light Source Inc., Saskatoon, SK) using the globar (silicon carbide) as the infrared source. The Bruker - IFS 66V/S spectrophotometer (Bruker Optics, Ettlingen, Germany) fitted with a deuteratedtriglycine sulphate (DTGS) detector was used for FTIR measurements. Root samples of S-Ck, R-Ck, S-Pb and R-Pb were prepared using the protocol described earlier with slight modifications. Root samples were dehydrated in a vacuum freeze drier (Labconco, Kansas City, MO) and ground into fine powders with a mortar and pestle. Approximately 2 mg of freeze dried powder were homogenized with 0.93 g of dry potassium bromide (KBr) with mortar and pestle, and made into a pellet under 8-ton pressure using a hydraulic press (Manual hydraulic press 15 Ton, Specac, Orpington, UK). Infrared spectrum was obtained in a transmission mode from each pellet, with KBr alone used as a background. Each IR spectrum was recorded in the mid infrared range of 4000-600 cm^{-1} wavenumbers at a spectral resolution of 2 cm^{-1}. For each sample, the spectrum value was an average over 64 scans against the background spectrum value (KBr alone, 128 scans). Baseline correction was applied to each normalized spectrum using a rubber-band correction (64 points) and vectors normalized using the OPUS software (version 7.0, Bruker Optics Inc., Billerica, MA). All FTIR spectra reported were averaged over two replicates (measurements of 10 pellets per replicate). The FTIR peaks reported in **Table 1** were determined using the Quick Peaks routine in OriginPro with local max settings at 0% threshold height, no baseline and the area at Y = 0.

Table 1. The assignment of main functional groups based on FTIR spectra of susceptible (S) and resistant (R) root samples. Wave numbers presented in the table are the mean of vibrational range.

Wavenumber (cm^{-1})	Absorption peak location and assignment	Components
3410	-OH groups, NH stretching	Proteins (Amide A)
2927	C-H stretch (asym.) of CH_2	Lipid acyl chains
1740	C=O stretching: carbonyl ester compounds	Pectin
1650	-C=O- and -C=N- stretching	Proteins (Amide I)
1548	N-H bending and C-N stretching	Proteins (Amide II)
1518	CH_2 and CH_3 methylene chain stretching	Lignin
1246	C-O, -CH_2- stretching and bending, P-O stretching	Hemicellulose
1151	C-O-C asymmetric stretching, PO_2 stretching	Cellulose
1061	C-O-C symmetric stretching	Cellulose

A second derivative was applied to the spectra to compare R and S samples and evaluate the effect of infection on the composition of cell wall. The integrated absorption band area was determined using the OPUS integration method C [34]. Secondary protein structure of both R

and S with/out inoculation was assessed following the protocol described by Lahlali et al. [34]. ANOVA and LSD (SAS institute, Cary, NC) were used to determine differences between inoculated and non-inoculated samples at $P \leq 0.05$. Raw spectral data were imported into the Unscrambler software (Version 10.1; CAMO Software AS, Norway) to extract possible differences between R and S. Before the principal component analysis (PCA), spectral data were subjected to Multiplicative Scattering Correction (EMSC) and second derivatives to Goly-Savitzky algorithm using 9 smoothing points. PCA was performed on all datasets with each FTIR wavelength considered as an equally weighted variable.

Transcription of cell-wall and lignin-related genes in canola root samples: The objective of this experiment was to assess the transcriptional levels of selected genes involved in lignin biosynthetic pathways [Phenylalanine ammonialyase (*BrPAL1*, *BrPAL2*, and *BrPAL3*), 4-coumarate (CoA ligase-*Br4CL* and cinnamate-4-hydroxylase-*BrC4H*), hydroxycinnamoyl (cinnamyl alcohol dehydrogenase-*BrCAD* and cinnamoyl-CoA reductase-*BrCCR*) as well as other genes involved in cell wall metabolism (Glutathione-S-transferase-BrGST and Xyloglucanendo-transglycosylate-BrXTH) induced by the *P. brassicae* inoculation using a StepOne® Plus quantitative RT-PCR (qPCR) system (Life Technologies, Mississauga, ON). RNA samples from both inoculated and non-inoculated roots were prepared as described earlier. Complementary DNA was synthesized using the Invitrogen Super Script III First-strand Synthesis system from 1 μg of total RNA, and PCR conducted using the Power SYBR green master mix (Life Technologies) following manufacturer's instruction. Cycling conditions were 95°C for initial 10 min followed by 40 cycles of 15 s at 95°C, 30 s at 50°C and finally 30 s at 60°C.Melt-curve profiling and agarose gel electrophoresis were conducted to confirm the specificity of reaction and absence of primer dimers. The actin gene Bra037560 was used as an endogenous control to normalize the expression level of target genes for its consistent level of expression among samples. The relative expression data were analysed using the StepOne® software V2.2.2 (Life Technologies).The whole transcription experiment was run twice (two biological replicates), with three technical replicates for each of the sample groups in a repetition. ANOVA and LSD ($P < 0.01$) were performed using the software Statistical Product and Service Solutions (V20.0; IBM, Markham, CA) for transcription quantity with each of the genes examined.

***Characterization of host cellular defense responses*:** This study is designed to differentiate temporal and spatial disruptions of infection by *P. brassicae*. The 2-3 selected CR genotypes and commercial CR cultivars from two seed companies wereexamined in a time course with a CS genotype to understand host cellular reactions at different infection stages, including epidermal infection, cortical infection and disease symptom development. Histochemical methods plus disease severity assessment (Deora et al. 2012) were used. The information from the above studies will improve our understanding of CR mechanisms, differentiate action modes associated

with different CR genes, and facilitate the selection of optimal CR genes for the development of CR canola germplasm.

Confocal Microscopy was used to exam the cell-wall lignification and the effect on clubroot resistance expression. Nile red (318.369 g mol^{-1}, Invitrogen) staining solution was produced by dissolving Nile red in methanol (1 mg mL^{-1}). Then a 15 µM staining solution was prepared by diluting the stock solution in water. The aqueous Nile red will be directly applied to a glass slide prior to placing root samples on the slide. Root samples were incubated in this solution for >5 min to stain the plasmodia of P. brassica. Aniline blue (0.1% (w/v) in 0.1 M K2HPO4 (pH 8.80) was used to stain callose in intact and sectioned root sample. Images are produced using strictly identical acquisition parameters (laser power, pinhole, detector gain, scanning speed, zoom factor, and resolution) for all samples. Aniline blue was applied to samples directly on the slide after staining with Nile red and were incubated at room temperature in the dark for 30 min prior to observation.

Root samples were taken at 10, 20 and 35 dpi; sections were taken over 2 cm of the main tap root below the hypocotyl. Root segments were embedded in FSC 22 (Leica) and frozen at −20°C in a Leica CM1860 cryostat microtome. Each frozen root piece was sectioned transversely into thin slices (15 µm) using the cryostat. 10 sites containing the objects of interest in each sample were photographed, for a total of 30 infection sites per time-course point per sample group. For each sample type, three biological replicates were used. Confocal microscopy was preformed using a Zeiss LSM 510 Meta inverted confocal microscope, with excitation wavelengths of 405 nm and 488 nm supplied from a UV light source and an Argon laser, respectively. To capture emission spectra of lignin autofluorescence with the excitation wavelength at 405 nm, the emission light was filtered through a 420 nm to 480 nm band pass filter. The image of lignin autofluorescence intensity was produced using strictly identical acquisition parameters outlined above for all samples. Each sample group had three biological replicates.

Table 2 Microscopic detection of *P. brassicae* life stages in canola roots inoculated with resting spore suspension of pathotype 3 or pathotype 5x.

Days after inoculation	Pathotype 3		Pathotype 5x	
	Westar	Double CR-gene line	Westar	Double CR-gene line
10	PL, ZS, CI	PL, ZS	PL, ZS, RCI	PL, ZS, CI
20	PL, ZS, H	PL	PL, ZS, H	PL
35	PL, RS, H	PL	PL, RS, H	PL, RS, H

PL: Plasmodium; **ZS**: Zoosporangia; **CI**: Cortex infection; **RS**: Resting spore; **H**: Hyperplasia;

***Introgression of clubroot resistance into B. napus and B. juncea canola*:** Once the candidate CR genes were chosen, based on the modes of action and/or resistance to different pathotypes, A-genome and B-genome CR genes will be introgressed into elite AAFC *B. napus*, *B. carinata* and industry *B. juncea* canola lines, respectively. MAS was used whenever the markers were sufficiently robust. Resistant lines developed were evaluated against targeted Pb pathotypes to ensure the effectiveness and consistency. Homozygous resistant materials were produced for tech transfer.

References

Audic S, Claverie JM. 1997. The significance of digital gene expression profiles. Genome Research 7: 986-995

Canola Council of Canada 2014: http://www.canolacouncil.org/news/new-clubroot-data-reinforces-need-for-vigilant-field-scouting/.

Cao T, Srivastava S, Rahman MH, Kav N N, Hotte N, Deyholos MK, Strelkov SE. 2008. Proteome-level changes in the roots of Brassica napus as a result of Plasmodiophora brassicae infection. Plant Science 174: 97-115

Deora A, Gossen B D, McDonald M R. 2012. Infection and development of Plasmodiophora brassicae in resistant and susceptible canola cultivars. Can. J. of Plant Pathol. 34: 239-247

De Vos RC, Schipper B, Hall RD. 2012. High-Performance Liquid Chromatography–Mass Spectrometry Analysis of Plant Metabolites in Brassicaceae. Methods in Molecular Biology, Clifton, NJ, 860, pp111-128

Gao F, Hirani AH, Liu J, Zheng Liu, Fu G, Wu C, McVetty PBE, Li GY. 2014. Fine mapping a clubroot resistance locus in Chinese cabbage. J. Amer. Soc. Hort. Sci. 139: 247–252.

Hasan MJ, Strelkov SE, Howard RJ, Rahman H, 2012. Screening Brassica germplasm for resistance to Plasmodiophora brassicae pathotypes prevalent in Canada for broadening diversity in clubroot resistance. Can. J Plant Sci 92:501-515

Hwang SF, Strelkov SE, Feng JI, Gossen BD, Howard RJ. 2011. Plasmodiophora brassicae: a review of an emerging pathogen of the Canadian canola (Brassica napus) crop. Mol. Plant Pathol. 13 : 105-113

Hwang SF, Howard RJ, Strelkov SE, Gossen BD, Peng G. 2014. Management of clubroot (Plasmodiophora brassicae) on canola (Brassica napus) in western Canada. (Invited Review). Can. J. Plant Pathol. 36 (Suppl. 1): 49-65.

Kaur P, Jost R, Sivasithamparam K., Barbetti MJ. 2011. Proteome analysis of the Albugo candida–Brassica juncea pathosystem reveals that the timing of the expression of defence-related genes is a crucial determinant of pathogenesis. J. Expt Bot. 62: 1285-1298

Ludwig-Müller J. 2009. Glucosinolates and the clubroot disease: defense compounds or auxin precursors? Phytochemistry Rev, 8: 135-148.

Ludwig-Müller J, Pieper K, Ruppel M, et al. 1999. Indole glucosinolates and auxin biosynthesis in Arabidopsis thaliana L. glucosinolates mutants and the development of the clubroot disease. Planta 208, 409-419.

Ludwig-Müller J, Scubert B, Pieper K, Ihmig S, Hilgenberg W. 1997. Glucosinolate content in susceptible and tolerant Chinese cabbage varieties during the development of the clubroot disease. Phytochemistry 44, 407-414.

Mortazavi A., Williams BA, McCue K, Schaeffer L, Wold B. 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nature Methods, 5: 621-628

Pedras MSC, Zheng QA, Strelkov S. 2008. Metabolic changes in roots of oilseed canola infected with the biotroph Plasmodiophora brassicae: phytoalexins and phytoanticipins? J. Agric. Food Chem. 56: 9949-9961

Peng G, Falk KC, Gugel RK, Franke C, Yu F, James B, Strelkov SE, Sheau-Fang Hwang S-F McGregor L. 2014a. Sources of resistance to *Plasmodiophora brassicae* (clubroot) pathotypes virulent on canola. Can. J. Plant Pathol, 36 (1) 89-99.

Peng G, McGregor L, Lahlali R, Gossen BD, Hwang SF, Adhikari, KK, Strelkov SE, McDonald MR. 2011. Potential biological control of clubroot on canola and crucifer vegetable crops. Plant Pathol. 60: 566-574.

Peng G, Pageau D, Strelkov SE et al. 2013. Assessment of crop rotation, cultivar resistance and Bacillus subtilis biofungicide for control of clubroot on canola. Acta Horticulturae (in press)

Peng G, Lahlali R, Hwang SF, Pageau D, Hynes RK, McDonald MR, Gossen BD, Strelkov SE. 2014b. Crop rotation, cultivar resistance, and fungicides/biofungicides for managing clubroot (*Plasmodiophora brassicae*) on canola. Can. J. Plant Pathol. 36 (Suppl. 1): 99-112.

Siemens J, Keller U, Sarx J et al. 2006. Transcriptome analysis of Arabidopsis clubroot indicate a key role for cytokinins in disease development. Mol. Plant Microbe Interact. 19:480-494

Strelkov SE, Hwang SF, Manolii VP, Cao T, Fredua-Agyeman R, Harding MW, Peng G, Gossen BD, McDonald MR, Feindel D. 2018. Virulence and pathotype classification of *Plasmodiophora brassicae* populations collected from clubroot resistant canola (*Brassica napus*) in Canada. Can J Plant Pathol. 40: (in press).

Wang F, Li L, Li H, Liu L, Zhang Y, Gao J, Wang X. 2012. Transcriptome analysis of rosette and folding leaves in Chinese cabbage using high-throughput RNA sequencing. Genomics 99: 299-307.

Williams PH. 1966. A system for the determination of races of *Plasmodiophora brassicae* that infect cabbage and rutabaga. Phytopathology. 56:624–626.

Results and comments

Initial assessment of CR candidates against pathotype 5x of *P. brassicae*

A total of 24 AAFC CR lines were screened against a mixture of three populations (L-G1, L-G2, and L-G3) of *P. brassicae* (Pb) pathotype 5x from Alberta fields where resistant cultivars failed in 2013. These Pb populations showed variable virulence against resistant canola cultivars, with L-G1 and LG2 being slightly more virulent on resistant varieties. The results showed that several CR genes/lines were resistant to the mixed 5x populations (**Table 3**). The data on several of the lines was not included (yellow highlight) due to poor seed germination and too small number of plants to assess. Further testing of selected candidates using a mixture of four 5x populations (DG-3 added) showed that the AAFC 01, 02 and 6 did better (**Table 4**) than in the three 5x populations showed in **Table 3**. Variations were seen among the CR candidates in response to different 5x populations; it appears that DG-3 is less virulent and, when mixed with other 5x populations, it seems to make CR candidates more resistant to pathotype 5x. It is clear that there are further variations among pathotype 5x populations.

The AAFC CR brassica candidates screened against the 5x populations included those carrying the CR genes *Rcr1, Rcr2, Rcr3, Rcr4, Rcr5* and *Rcr6* identified previously from *B. rapa* and *B. nigra*. These CR genes showed resistance to all other Pb pathotypes found in Canada earlier. Each of these CR genes has now been mapped, with robust markers developed. *Rcr1*(A03) and *Rcr3* (A08) are identified in *B. rapa*, and showed differential resistance to 5x; *Rcr1* is generally susceptible while Rcr3 showed resistance to certain 5x populations, especially to DG-3. *Rcr6* is from *B. nigra* (B genome) and showed resistance to all *P. brassicae* pathotypes or populations tested. Although the Rcr6 is highly resistant against different pathotypes, being a B genome material makes it a challenge to incorporate the CR gene into *B. napus* canola but may be more readily usable for development of clubroot resistant *B. carinata* or *B. juncea* canola.

Table 3 Screening of AAFC Brassica lines against a mixture of three pathotype 5x populations (*Plasmodiophora brassicae* L-G1, L-G2, L-G3, DG-3) in a greenhouse.

Resistant candidate	Total no. of plants	No. of plants in each category**				Index of disease (%)
		0	*1*	*2*	*3*	
ECD 05 [1]	36	0	0	0	36	100
AAFC 01	36	2	3	7	25	85.2
AAFC 02	36	0	2	2	32	94.4
AAFC 03	36	0	2	4	30	92.6
AAFC 04 [2]	36	25	8	3	0	13.0
AAFC 05	36	26	6	4	0	13.0
AAFC 06	36	24	2	3	7	26.9
AAFC 07	36	32	4	0	0	3.7
AAFC 08 [3]	-	-	-	-	-	-
AAFC 09	36	7	7	8	14	60.2
AAFC 10	36	2	2	6	27	88.0
AAFC 11	36	0	0	0	36	100
AAFC 12	36	0	4	4	28	88.9
AAFC 13	36	35	1	0	0	0.9
AAFC 14	36	4	13	14	15	79.6
AAFC 15	36	15	8	8	5	36.1
AAFC 16	-	-	-	-	-	-
AAFC 17	-	-	-	-	-	-
AAFC 18	-	-	-	-	-	-
AAFC 21	36	21	7	7	1	22.2
AAFC 22	36	0	1	20	15	79.6
AAFC 23	36	22	6	7	1	21.2
AAFC 24	36	14	3	8	11	48.1

[1] Universally susceptible control
[2] Green highlight indicates resistance relative to the susceptible control
[3] Yellow highlight: the line had too low germination to be assessed properly.

Table 4 Assessment of selected AAFC clubroot resistant Brassica genotypes against a mixture of four pathotype 5x populations (L-G1, L-G2, L-G3 & DG-3) in a greenhouse.

Resistance candidate	Total # of plants*	Number of plants rated in each category				Index of disease (%)
		0	1	2	3	
ECD 05 [1]	48	0	0	2	46	98.6
AAFC 01	56	51	0	1	4	8.3
AAFC 02	38	20	4	3	11	37.7
AAFC 05	20	14	2	1	3	21.7
AAFC 06	38	33	2	3	0	7.0
AAFC 14	68	30	8	6	24	45.1
AAFC 15	67	25	9	7	26	50.2

[1] Universally susceptible control

Resistance mechanisms associated with CR gene(s)

Leading-edge technologies were explored to decipher the mechanisms associated with specific CR genes, including transcriptome (RNA-seq), proteome, metabolome, fluorescent microscopy and synchrotron-based chemometrics. These technologies can be used to identify metabolic or signaling pathways relating to clubroot resistance, potentially differentiating the modes of action among different CR genes and guiding the deployment of CR genes, in pyramiding or rotation, to enhance the resistance durability. RNA-seq had been used to identify the molecular modes of action for the CR gene *Rcr1* before the start of this project, with jasmonic acids/ethylene and callose deposition identified as the key signaling and metabolic pathways in mediating clubroot resistance in *B. rapa*, whereas the salicylic acid pathways (a common host-defense mechanism) did not appear to be involved.

Proteome and metabolome analysis conducted during the project showed further depth of resistance mechanism of *Rcr1*. A shotgun proteomic approach was used for the proteome study. The roots were sampled at 14 days posted inoculation (pdi) using markers and the rate of root infection by *P. brassicae* was assessed by keeping additional plants until up to 42 dpi. All plants without *Rcr1*showed clubroot while none of the plants carrying the CR gene had any sign of root swelling (**Fig 1A**). Based on the reference peptide sequences of *B. rapa* Chiifu-401, a total of 2,002 and 1,859 proteins, respectively, were identified in roots carrying and not carrying *Rcr1* (susceptible CS) (**Table 5**), with a total of 2,229 non-redundant proteins. Cluster analysis, based on the correlation between the expression profile among replicates, showed that these replicates were separated clearly depending on the presence/absence of *Rcr1* (**Fig 1B**). This indicates high reproducibility of data. Statistical analysis (*t* test, $p \leq 0.05$) of the 2,229 total proteins identified 527 differentially accumulated proteins (DAPs), with 296 being increased and 231 decreased in

resistant samples relative to susceptible ones (**Fig 1C**). Of the 527 DAPs, 523 could be annotated using Blast2GO, based on gene ontology (GO) terms, and sorted into major biological processes (**Fig 1D**), including signaling, localization, growth, biological regulation, response to stimulus and cellular, developmental or metabolic processes. The results indicate that the perception of Pb and subsequent activation of defense responses is triggered likely via a novel signaling pathway acting in a calcium-independent manner through a cascade of mitogen-activated protein kinases requiring the ubiquitin-26S, a proteasome reported frequently in plant cold tolerance responses. It is interesting that similar pathways are activated when plants respond to clubroot infection and cold stress.

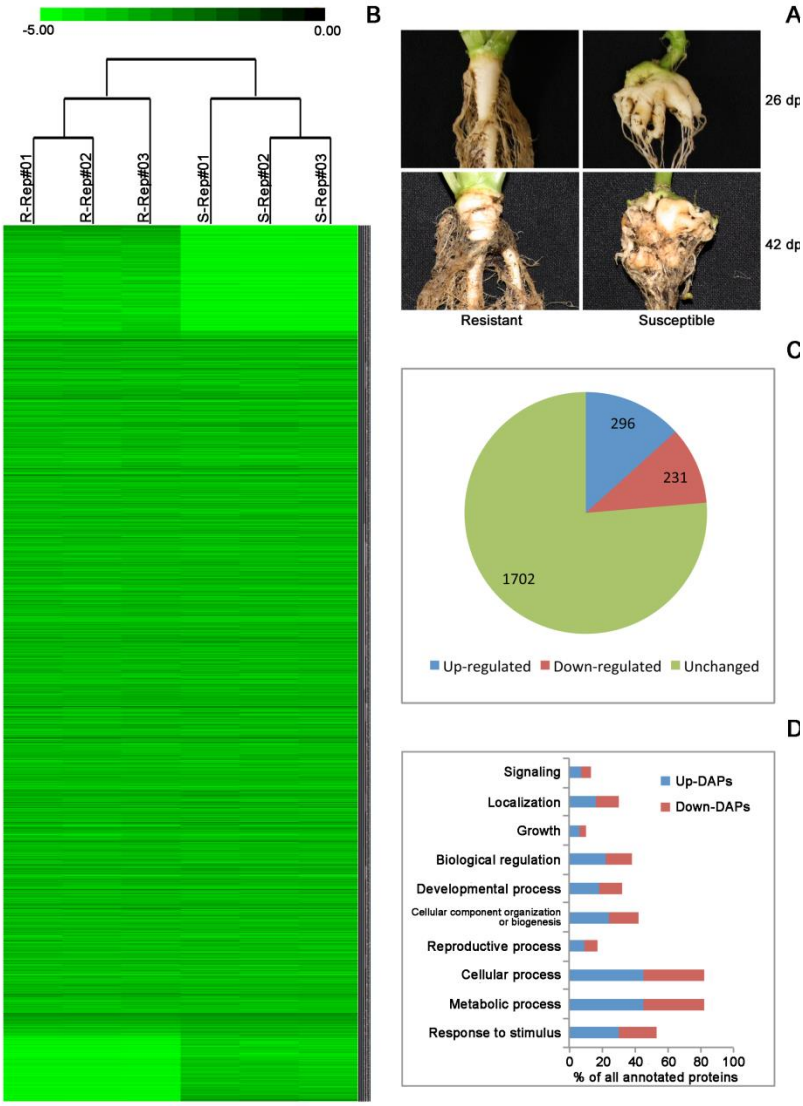

Fig 1. A) Roots of *B. rapa* plants carrying/not carrying *Rcr1*at 26 and 42 dpi with *P. brassicae*. Club symptoms did not develop on plants carrying *Rcr1*. **B**) A color map of 2,229 non-redundant proteins found in three biological replicates of root samples carrying or not carrying *Rcr1*. The darker color represents greater abundance based on spectral abundance factor values. R: Resistant; S: Susceptible; Rep: Replicates. **C**) Among the 2,229 non-redundant proteins, 527 were differentially accumulated with 296 being up-regulated and 231 down-regulated in the samples carrying *Rcr1*. **D**) Functional classification of differentially accumulated proteins (DAPs).

Table 5. Summary of proteins identified using GPM search.

Genotypes	No. identified proteins			ρ value[1]/FPR[2] (%)			No. proteins
	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	
Resistant[3]	3297	3302	3602	93/0.71	92/0.70	93/0.70	2,002
Susceptible[3]	3228	3187	3153	93/0.71	93/0.67	93/0.70	1,859

[1] *p* value is calculated by GPM software to evaluate the quality of the search. The value ranges from 0 (indistinguishable from purely stochastic results) to 100 (putatively all true positives).

[2] The FPR is the false positive rate.

[3] Plants carrying (resistant) and not carrying (susceptible) the CR gene *Rcr1*, respectively.

The proteomic results indicate that the resistance to clubroot conferred by *Rcr1* is caused by the perception of infection through an unique MAPK cascade, which may require the ubiquitin-26S proteasome linked frequently to cold-stress tolerance. A range of biological processes were also identified in resistant Rcr1-carrying root samples where they were either up-regulated for host-defense responses or down-regulated for the metabolism favoring disease development. The former would include higher ROS accumulation, breakdown of sulfur-containing glucosinolates and lignin biosynthesis, whereas the latter include decreased glycolysis and arginine catabolism. This is the first proteomic study on clubroot resistance relating to a specific CR gene; it provides insights into the resistance modes of action for *Rcr1* by comparing the results with those from transcriptomic analysis, and identifies additional pathways for clubroot resistance. This work has been published in the *Frontiers in Plant Science* (Song et al. 2016).

Metabolome profiling was performed on an LTQ-Orbitrap MS Classic system equipped with a Luna C18 reverse phase column (Aliferis et al., 2012). Biomarker discovery was based on the Student *t-test* or unpaired parametric *t-test* and putative identification of metabolite was based on targeted searches against an in-house library for canola at the McGill University. Metabolites of interests were further quantified by reversed-phase LC-MRM/MS on a UPLC column. Authentic compounds from commercial sources were employed to construct linearly-regressed standard curves for quantification of targeted metabolites. The partial least square-discriminant analysis (PLS-DA) of samples showed clear discrimination of metabolic profiles between plants carrying and not carrying Rcr1 or with/without pathogen inoculation (**Fig 2**). A total of 153 differentially accumulated metabolites (DAMs) were identified in plants carrying the CR gene relative to its non-inoculated control, while 210 DAMs were found in inoculated plants without *Rcr1* relative to its non-inoculated control. These DAMs can be divided into many types of chemical groups, including phenol, alcohol and fatty acid etc. The accumulation of jasmonic acids as well as the metabolites involved in its signal transduction, was significantly increased as determined by the

Direct Infusion Mass Spectrometry assay (**Table 6**). Furthermore, a strong increase of an inactive form of auxin, methyl-IAA, was also observed. Additionally, several flavonoids, including the phytoalexin caulilexin C, were also indicated with increased quantities. Many of these flavonoids have shown anti-microbial activities against other phytopathogens. The increased accumulation of both phytohormone and the caulilexin C were subsequently confirmed during the targeted analysis.

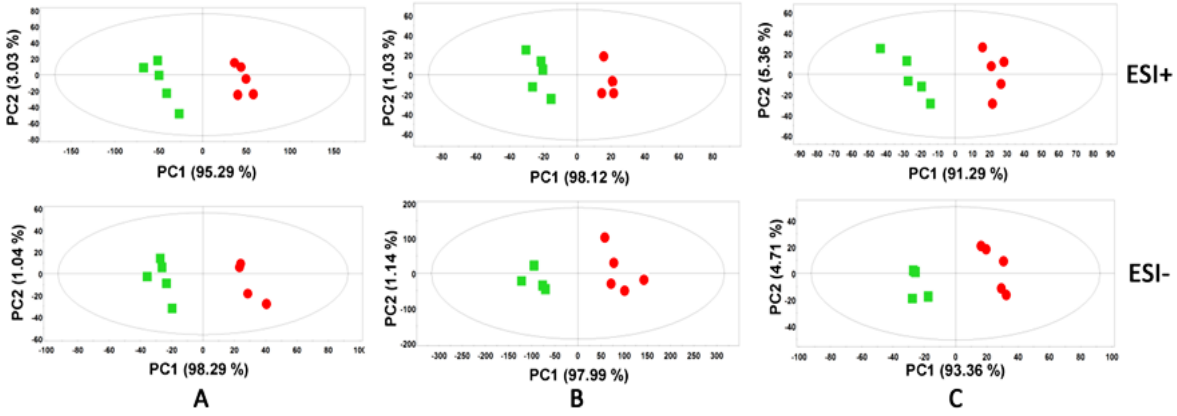

Fig 2. **A**) PLS-DA plots. Plants carrying no *Rcr1*: Non-inoculated (red) vs inoculated (green); **B**) Plants carrying *Rcr1*: Non-inoculated (red) vs. inoculated (green); **C**) Inoculated plants without *Rcr1* (red) vs with *Rcr1* (green).

Table 6. Selected differentially accumulated metabolites (DAMs) identified from plants carrying *Rcr1* (DIMS assay).

DAMs	Chemical group	*p*-value	Non-inoculated	Inoculated	Fold change
Jasmonate	Fatty acid	0.003	152	1101	+7.25
Jasmonoyl-L-isoleucine	Conjugate	0.044	132	1436	+10.88
Caulilexin C	Heterocyclic compound	0.02	6655	13473	+2.02
Methyl-IAA	Aromatic compound	0.039	123852	238551	+1.93

Synchrotron-based fourier transform mid Infrared (FTIR) spectroscopy identified clear changes in the cell-wall composition associated with the resistance conferred by *Rcr1*, especially the increase in lignin composition. Sisignificant differences in the absorption of OH, CH_2 and CH_3, and fingerprint regions (1800-800 cm^{-1}) where cell wall constituents could be assigned and distinguished (**Fig 3**). The three components PC-1, PC-2 and PC-3 explained, respectively, 59%, 20% and 7% of the variation in canola root samples, and distinguished between substantially infected (S) and little-infected (R) roots by *P. brassicae* (**Fig 3A, 3B**). According to the loadings plot (**Fig 3C**), the cell-wall components of root were changed for plants carrying *Rcr1*, including lipids, proteins, lignin, phenolics, cellulose/hemicellulose and carbohydrates. The RT-qPCR assay found that *P. brassicae* inoculation generally enhanced the expression of genes involved in lignin pathways, relative to those of non-inoculated, but the level of increase was substantially more pronounced in plants carrying the CR gene *Rcr1*, especially the higher expression of *BrPAL1*(**Fig 3D**). The up-regulation of this *PAL* gene is indicative of an inducible defence response conferred by *Rcr1*; the activation of this basal defence gene via the phenylpropanoid pathway contributes to the clubroot resistance.

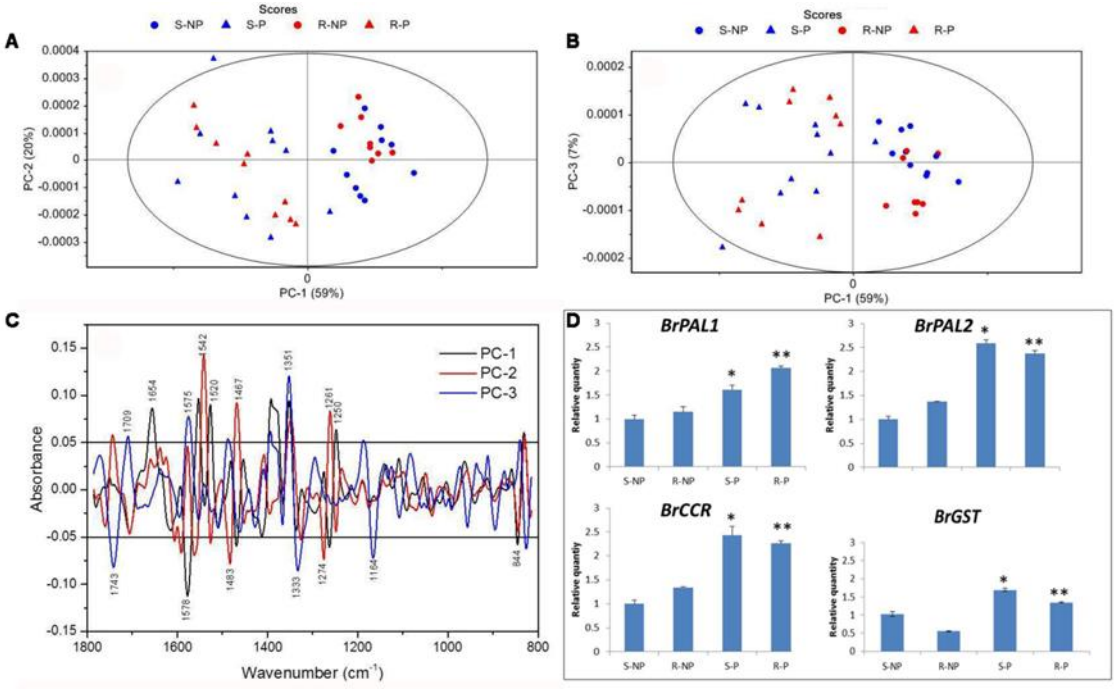

Fig 3. **A**) and **B**) FTIR analysis and qRT-PCR of selected lignin genes. Principal component analysis (PCA) scores plots and **C**) the loading plots. **D**) Control (NP) and inoculated (P) roots of R and S plants in the fingerprint spectral region (1800-800 cm^{-1}). Transcriptional levels of selected lignin biosynthetic genes in qRT-PCR.

Effect of single vs double CR-gene canola lines against pathotype 5x

Although several canola lines carrying a single CR gene showed substantial resistance against mixed populations of pathotype 5x, further test of these lines against the populations L0G2 and L-G3 separately showed high susceptibility (**Fig 4**). These two 5x populations appeared highly virulent to most of the single CR-gene canola lines/varieties assessed. Through the collaboration with seed companies, hybrid canola (*B. napus*) lines carrying double CR genes were generated and tested against the same virulent populations of 5x. The results showed that crosses with the DH canola line carrying CRB (A8) as a female, and line carrying *Rcr1* (A3) or CRM (A3) as a male would result in moderate resistance to both 5x populations, while the reciprocal crosses of these lines failed to produce the same effect (**Fig 5**).

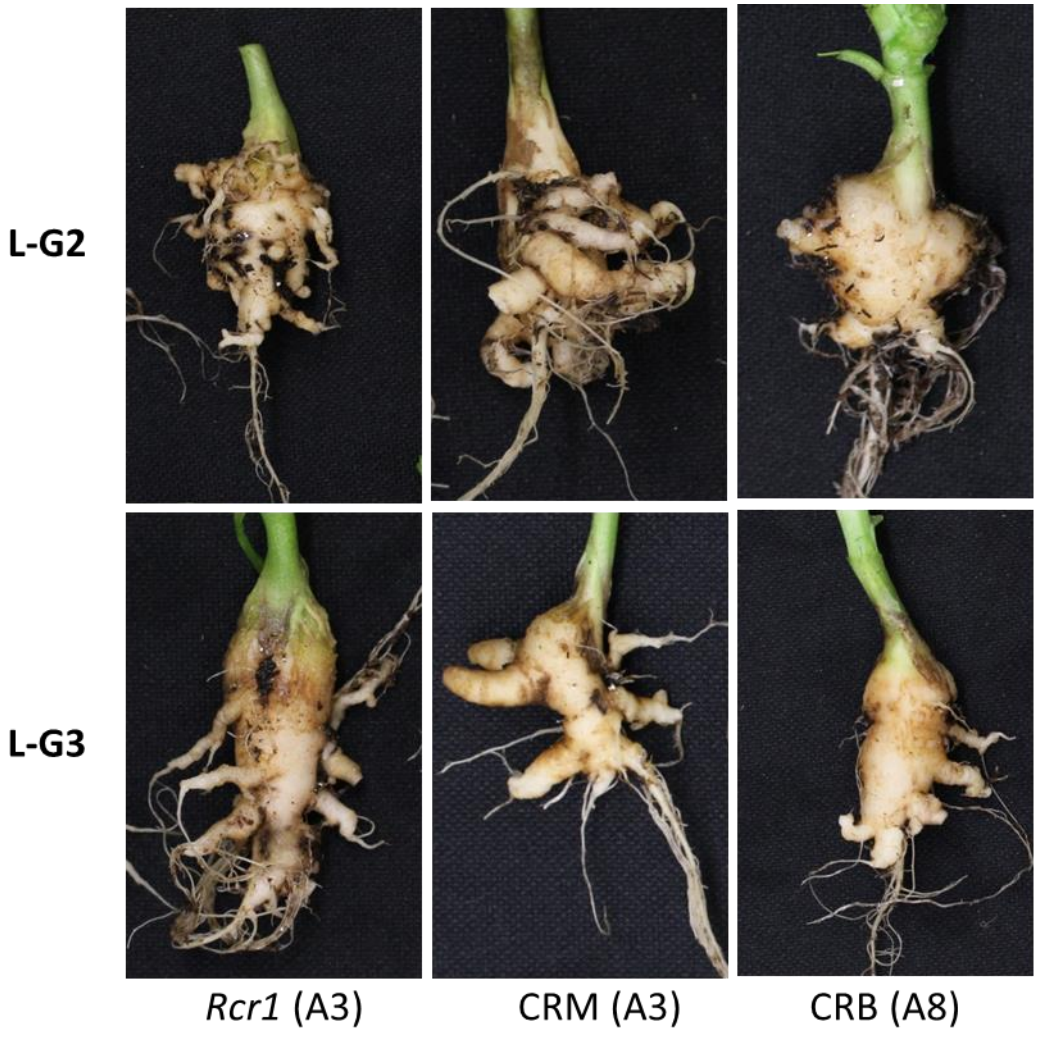

Fig 4. Clubroot severity caused by pathotype 5x (L-G2, L-G3) of *Plasmodiophora brassicae* on canola lines carrying a single resistance gene 5 weeks after the inoculation in greenhouse.

The genetic basis for the differential resistance of reciprocal crossing is not yet well understood; there seem to be cytoplasmic factors associated with the line carrying CRB that can interact with *Rcr1* or CRM in the nuclear to achieve a moderate level of resistance. The question is: will these lines be of real value in providing the resistance against all pathotype variants (Stelkov et al. 2018) and more durable than single CR-gene varieties when exposed repeatedly to the same population of *P. brassicae*? This work is being continued in a separate study also supported by SaskCanola and WGRF.

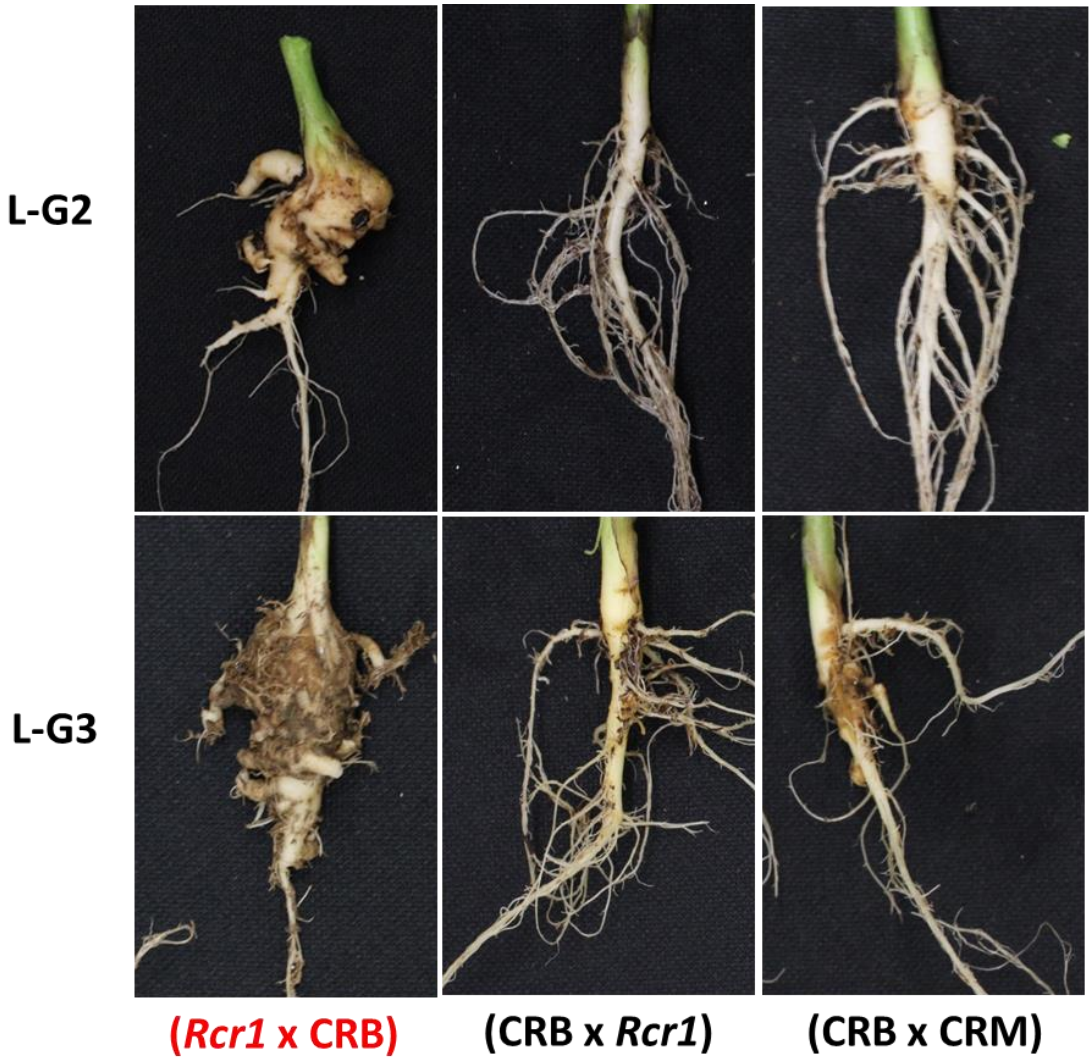

Fig 5. Clubroot severity caused by pathotype 5x (L-G2, L-G3) of *Plasmodiophora brassicae* on canola lines carrying double CR genes 5 weeks after the inoculation in greenhouse. Note the line on the left (*Rcr1* × CRB) carried the same CR genes as the line in the middle (CRB × *Rcr1*), but these two lines are derived from reciprocal crossings.

Microscopic examination showed differential clubroot infection in the roots of these two double CR-gene lines when compared to those of single CR-gene lines or susceptible control Westar; plasmodia or zoosporangia were observed in epidermal cells of both double CR-gene and single CR-gene/Westar lines/cultivars, but the percentage of cells with the presence of these pathogen structures was substantially lower in resistant double CR-gene lines (**Fig 6B**). In the cortical tissue of resistant roots, the incidence of plasmodia and zoosporangia was even much lower than in epidermal cells or not observed (**Fig 6C, 6D**). These results indicate that these CR lines, despite a moderate level of resistance, effectively reduce cortical infection and the minor root swelling symptoms may be caused by merely limited infection of the epidermal layer.

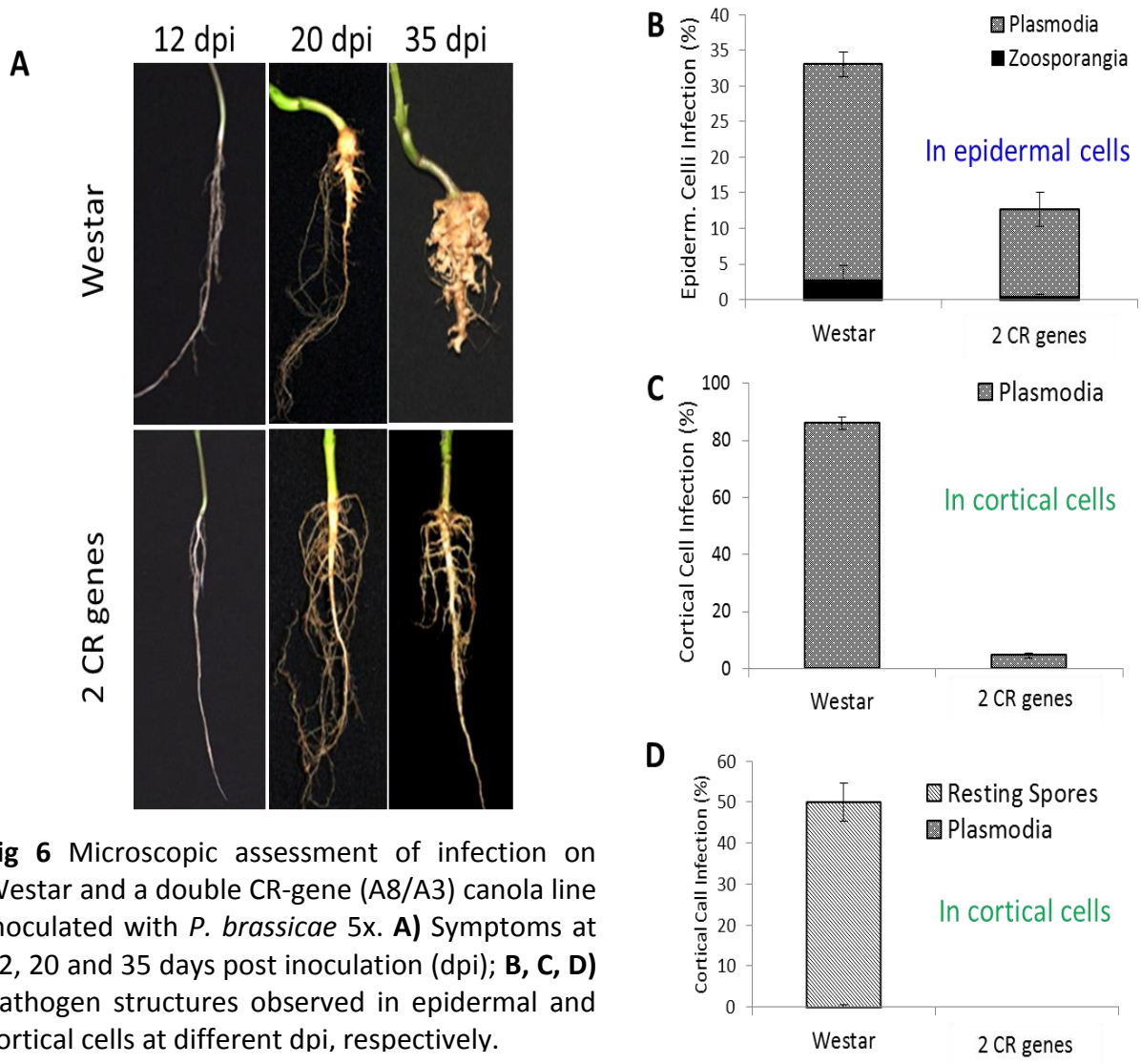

Fig 6 Microscopic assessment of infection on Westar and a double CR-gene (A8/A3) canola line inoculated with *P. brassicae* 5x. **A)** Symptoms at 12, 20 and 35 days post inoculation (dpi); **B, C, D)** pathogen structures observed in epidermal and cortical cells at different dpi, respectively.

At 12 to 35 dpi, autofluorescence of cell wall lignification was evident with vascular cells, even without the pathogen inoculation (**Fig 7**). When pathotype-3 inoculum was applied, there was stronger lignification of parenchyma cell wall in roots of double CR-gene lines relative to those of Westar, and this increased lignification was accompanied by complete resistance to the pathotype 3. When inoculated with pathotype 5x, similar lignification was observed in the parenchyma tissue of double CR-gene lines, but more noticeably after 20 dpi (**Fig 8**). Less or little lignification could be observed in the parenchyma tissues of non-inoculated or Wester roots. This indicates that increased lignin production is associated with clubroot resistance conferred by the double CR-gene lines against pathotype 3 and pathotype 5x. In transcriptome and synchrotron-based FTIR spectroscopic analyses, the importance of cell-wall modification including callose deposition and increases in lignin and phenolics was strongly indicated with the CR gene *Rcr1* in mediating clubroot resistance. The molecular mechanisms of double CR-gene lines are being investigated.

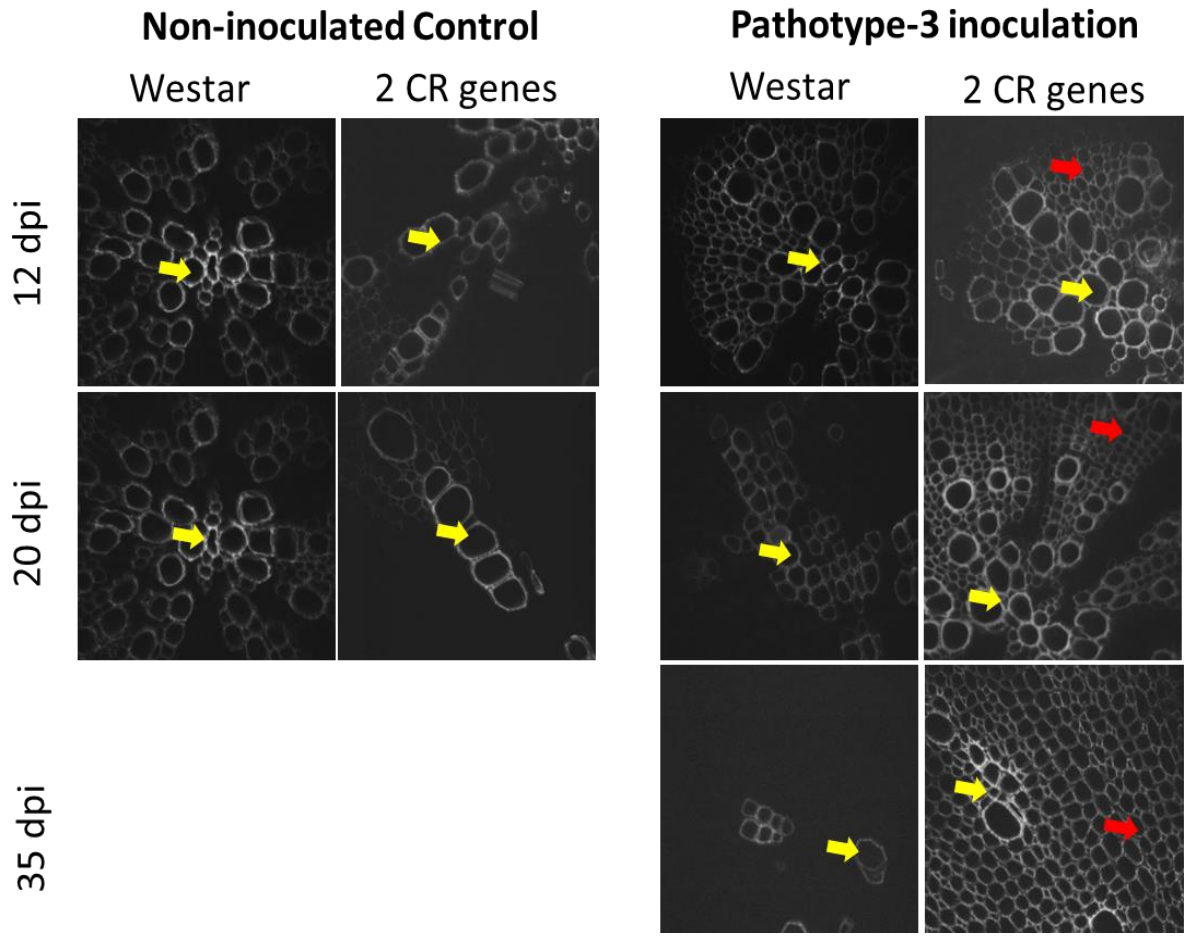

Fig 7. Lignification intensity of cell walls of CS and CR (A8/A3) canola roots inoculated with pathotype 3 of *P. brassicae*. Red arrows indicate lignified parenchyma cell walls, and yellow arrows show lignified vascular elements.

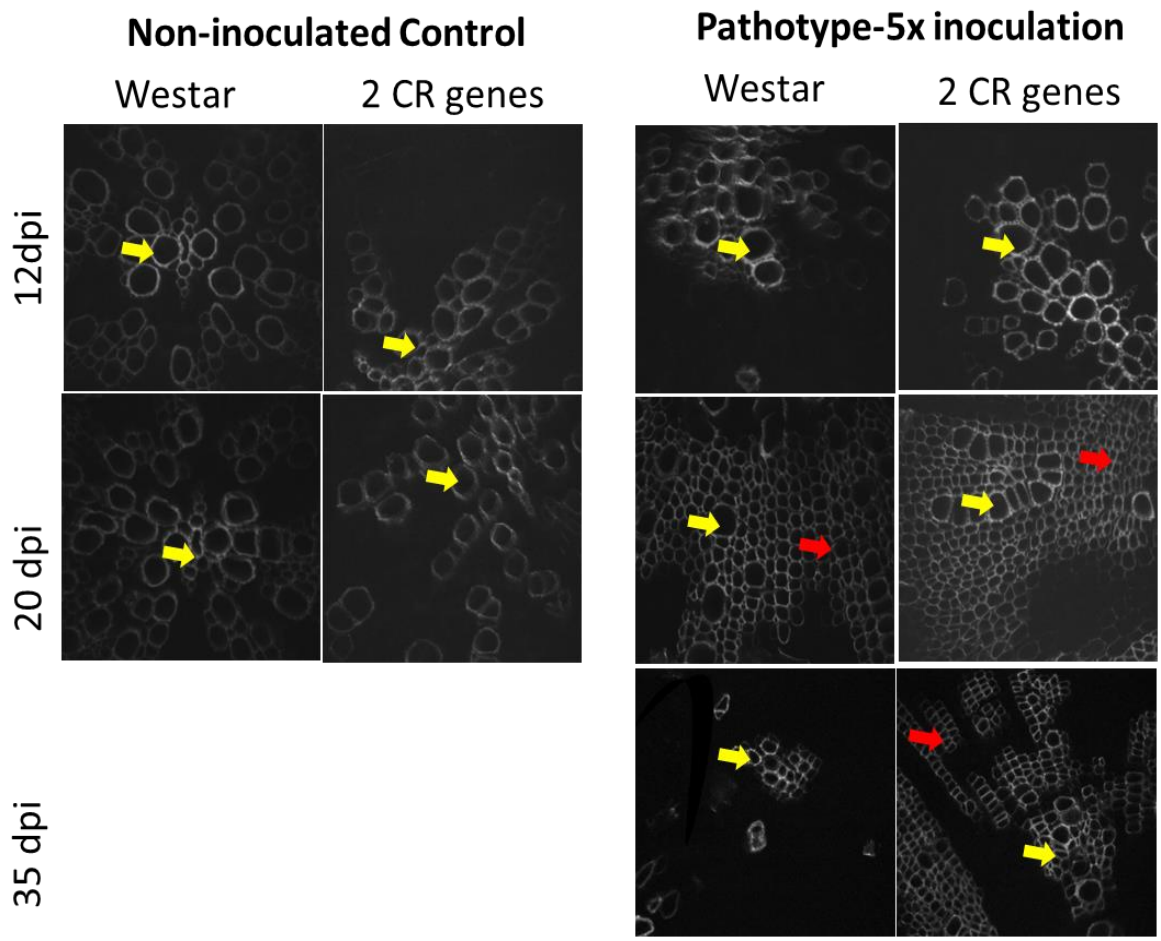

Fig 8. Lignification intensity of cell walls of CS and CR (A8/A3) canola roots inoculated with pathotype 5x of *P. brassicae*. Red arrows indicate lignified parenchyma cell walls, and yellow arrows show lignified vascular elements.

Developing canola germplasms carrying different CR genes

Using Bulked sergeant RNA sequencing, gene-specific SNP markers were developed for the CR genes *Rcr1*, *Rcr2*, *Rcr3 Rcr4* and *Rcr6* with differential efficacy against the pathotypes of *P. brassicae* found in Canada, including the 5x. MAS was used to incorporate these CR genes into *B. napus* (*Rcr1*, *Rcr2*, *Rcr3, Rcr4*), *B. carinata* (Rcr6) and *B. juncea* (*Rcr1* and *Rcr6*). All these CR genes are in elite AAFC breeding lines with stable homozygous BC5 seed produced for tech transfer, except *B. juncea* which was more challenging during inter-specific crossing. Very low fertility rates occurred when *B. juncea* lines were crossed with *B. rapa* (*Rcr1*) or *B. nigra* (*Rcr6*). *Rcr1*(A3), *Rcr2* (A3) and *Rcr3* (A8) have been pyramided into commercial canola hybrids for multi-genic approaches and some of these hybrids have been in field testing in Alberta since 2016.

The new CR canola germplasm included: single CR genes on A2, A3 and A8 chromosomes of A genome of Brassica, a single CR gene on B genome, and multi-genic germplasms carrying CR genes on A3 and A8. The resistant *B. rapa* line T19 was crossed with the *B. oleracea* cultivar Tekila to produce resynthesized *B. napus* line which showed resistance to each 5x populations tested. This resynthesized line showed the potential carrying multiple CR genes against the 5x and other pathotypes, and is being subjected to DH line production for further molecular characterization. The CR gene *Rcr6* (B genome) was promising against all pathotypes or variants found in Canada, but it can be more challenging to move it into *B. napus* (AC genome) without sacrificing the yield and quality of canola varieties substantially.

Some of these newly developed CR canola germplasms have been provided to breeding companies via non-royalty agreements. This approach accelerates tech transfer, and helps increase the diversity of CR genes in development of new resistant canola varieties. Those of CR genes on A3 chromosome appeared less effective when used alone, but reduced clubroot development by >80% when combined with a CR gene on A8. The CR gene on A2 appeared highly effective against 5xs, but not so much to pathotype 3. Staking of CR genes on A2 and A3 may be of value in terms of broadening the resistance against more pathotypes and new crosses will be made to stake these two CR genes.

It is becoming clearer that pathotype 5x is a mixed group of races not separable with the current host differentials. There will likely be further variants within the group, so it would be useful to decipher resistance mechanisms with different CR germplasms developed against the most aggressive populations of 5x for effective deployment of CR genes. The work under this project helped equip us with more advanced technologies, including transcriptome, proteome, metabolome and synchrotron for studying resistance mechanisms. A comparative study of single and multi CR-gene lines with differential efficacy against the 5x and other pathotype variants will provide more information for CR gene selection and deployment. A RFP process will be used for tech transfer of *Rcr6* to the industrial oil sector (*B. carinata*), and this would be the first B-genome CR gene to be used in canola. Although the introgression of *Rcr6, Rcr1* and *Rcr3* (both on A3) into *B. juncea* has been a challenge, resynthesized CR *B. juncea* (*B. rapa* × *B. nigra* –*Rcr6*) looked more promising, with a stable CR *B. juncea* line obtained. The versatility of this juncea line against different pathotypes of P. brassicae is being assessed and once confirmed, will be crossed with elite commercial *B. juncea* canola lines to tech transfer. Elite black-seeded AAFC *B. napus* lines carrying *Rcr1* (A3) and *Rcr3* (A8) have been field tested in a clubroot infested breeding nursery. A yellow-seeded elite AAFC *B. napus* breeding line is now at homozygous BC5F2 stages, and ready for field testing in 2018. Stable *B. rapa* canola germplasm carrying *Rcr1* has also been produced. Although the acreage of *B. rapa* canola has been declining in most areas on the prairies, the production is concentrated mostly in the Peace region, AB. Due to the discovery of clubroot in the region in 2017, there may still be the need for tech transfer of CR *B. rapa* canola germplasm. There needs and opportunity will be explored further through discussions with *B. rapa* canola seed suppliers.

Outputs/Deliverables

New/improved genetic materials

- Clubroot resistant *B. napus* germplasm carrying the CR genes *Rcr1, Rcr2, Rcr3* and *Rcr4* have been transferred to seven breeding companies for clubroot resistance breeding. *Rcr3* and *Rcr4* showed differential resistance to some of the pathotype 5x and other variant populations.
- *B. napus* germplasm carrying double CR genes (A3, A8) has been produced through the collaboration with Crop Production Services. Two of the double CR-gene lines showed a moderate level of resistance to the most virulent pathotype 5x populations (L-G2 and L-G3) consistently. Their efficacy against other pathotype variants and potential for durable disease resistance is being assessed.
- An elite line of *B. carinata* carrying homozygous CR gene *Rcr6* has been stabilized and can be used for commercial variety development. Genetic markers have been validated for MAS.
- Elite AAFC *B. napus* lines (black- and yellow-seeded) carrying the CR gene *Rcr1*or *Rcr3* have been produced and field testing has been carried out with the black-seeded lines. These CR genes can be pyramided in commercial breeding lines for canola hybrids with resistance against a broader range of pathotypes.

Scientific publications

Lahlali1 R, Song T, Chu M, **Yu F**, Kumar S, Karunakaran C, **Peng G**. 2017. Evaluating changes in cell-wall components associated with clubroot resistance using fourier transform infrared spectroscopy and RT-PCR. *Int. J Mol. Sci*. 18(10), 2058.

Song T, Chu M, Lahlali R, **Yu F**, **Peng G**. 2016a. Shotgun label-free proteomic analysis of clubroot(*Plasmodiophorabrassicae*) resistance conferred by the gene *Rcr1* in Brassica rapa. *Frontiers in Plant Science* 7: 1013.

Song T, Chu M, Lahlali R, Karunakaran C, **Yu F**, **Peng G**. 2016b. Using "omics" approaches to decipher resistance mechanisms of *Rcr1*. In: *Proc. Brassica 2016*. Oct. 3 – 6.

Yu F, Zhang XG, Huang Z, Chun MG, Song T, Chang A, Deora A, Chen Q, Zhang Y, McGregor L, Falk KC, Gossen BD, McDonald MR, Jing MY, **Peng G**. 2016. Identification of SNP markers associated with clubroot resistance gene *Rcr1* through bulked segregant RNA sequencing. *PLoS One* 11(4): e0153218.

Yu F, Zhang XG, **Peng G**, Falk KC, Strelkov SE, Gossen BD. 2017. Genotyping-by-sequencing reveals three QTL for clubroot resistance to six pathotypes of *Plasmodiophora brassicae* in Brassica rapa. *Scientific Reports* 7: 4516.

Zhen Huang, **Gary Peng**, Xunjia Liu, Abhinandan Deora, Kevin Falk, Bruce D. Gossen, Mary Ruth McDonald, **Fengqun Yu**. 2017. Fine mapping of a clubroot resistance gene in Chinese

cabbage using SNP markers identified from bulked segregant RNA sequencing. *Frontiers in Plant Sci* 8: 1448.

Information events

Peng G. 2018. Approaches to improve the understanding of clubroot resistance mechanisms and generational resistance durability. International Workshop on Brassica Genomics, March 26-28, Wuhan, China.

Yu F. 2018. Use of resistance from black mustard for control of clubroot and blackleg in Canola. International Workshop on Brassica Genomics, March 26-28, Wuhan, China.

Peng G. 2017. Characterizing & developing new resistant sources for management of clubroot in canola. Canola Discovery Forum. December 5-6, Saskatoon, SK.

Peng G. 2017. Using "omics" to decipher mechanisms and guide deployment of clubroot resistance. Presentation at Int. Clubroot Workshop, Apr 24-27, Wuhan, China

Yu F. 2017. Genotyping by sequencing – a new approach for characterizing clubroot resistance. Presentation at Int. Clubroot Workshop, Apr 24-27, Wuhan, China

Lahlali R, Song S, Chu M, Yu F, Kumar S, Karunakaran C, **Peng G**. 2016. Cell wall components play a role in the clubroot resistance mediated by the gene *Rcr1*. Poster presented at Am. Phytopathol. Society Ann. Meeting, Tampa, FL, USA.

Song T, Chu M, Lahlali R, Karunakaran C, Yu F, **Peng G**. 2016. Using "omics" approaches to decipher resistance mechanisms of *Rcr1*. A poster presented at Brassica 2016. Oct. 3 – 6, Melbourne, Aus.