Agriculture et Agroalimentaire Canada | Agriculture and Agri-Food Canada
Saskatoon Research and Development Centre

Enhancing the durability of clubroot resistance with multiple genes

(Final Project Report –AGR-13417, WGRF VarD1603, SCDC 2015-64)
July 6, 2019

Dr. Gary Peng

Executive Summary

Clubroot continues to spread on the Canadian prairies, posing serious threat to canola production. Cultivar resistance is the key to clubroot management, but the 17 "new" pathotypes identified recently in Alberta all appeared virulent to current resistant cultivars in the marketplace. New resistance sources, as well as strategies, are required to address this challenge. Earlier work indicated that none of the single CR genes in our arsenal would be able to control all of the new pathotypes identified (2B, 3A, 3B and 5X etc.). It was not known if stacking clubroot resistance (CR) genes would provide more sophisticated mechanisms for a broader range of efficacy and greater durability against different infestation on the prairies, including new pathotypes, as well as high and low levels of the predominant pathotype 3H.

This study assessed the efficacy and durability of canola lines carrying single and multiple CR genes under simulated intensive canola-growing conditions. All lines selected are resistant to the old pathotypes 2, 3, 5, 6 and 8, but the efficacy against the newly identified pathotypes was unknown. Additionally, resistance durability against the predominate pathotype 3H (old pathotype 3) under heavy (Alberta) and lighter (Saskatchewan and Manitoba) infestation was also investigated to better understand potential risks of resistance erosion associated with the deployment of CR genes in different regions. Potential interactions of stacked CR genes were examined using transcriptome analysis to identify unique molecular mechanisms associated with multi-genic lines. The study was intended to provide insights into resistance durability for multi-genetic CR strategies.

The study revealed that CR genes on chromosome A8 (*CRB*) are effective to the predominant pathotype 3H (old pathotype 3) but only partially resistant to two of the new pathotype 5X populations, and susceptible to the population L-G3. When combining *CRB* with one of the CR genes on chromosome A3 (*Rcr1* or *CRM*), moderate resistance was seen against each of the 5X populations, as well as to other old pathotypes. This shows that the range of resistance can be increased by stacking two CR genes of different modes of action. Many genes involved in pathogen associated pattern-triggered and effector-triggered immunity were strongly activated in canola lines carrying the two CR genes in response to infection by 5X. Despite an intermediate level of resistance, the resistance appears quite durable after five generational cycles of exposure to the same 5X population, with disease severity index (DSI) generally <30%. This highlights the value for using the multi-genic approach for CR efficacy and durability. More work is warranted to include additional new pathotypes to validate this multi-genic strategy for resistance durability. Against pathotype 3H, a single CR gene loses the resistance gradually likely due to the presence of virulent pathotypes in the inoculum, especially when exposed to high initial inoculum levels (10^7 spores/g soil). The resistance erosion is noticeably slower under lower inoculum pressure (10^4 spores/g). This emphasizes the value of extended crop rotation (>2-year break from canola) to reduce the load of resting spores in heavily infested fields; this can assist in the performance and durability of clubroot resistance.

Background

Clubroot (*Plasmodiophora brassicae* Woronin) remains a threat to canola production in western Canada, with increased number of infested fields reported every year, especially in Alberta. New pathotype variants reported in Alberta virulent to all clubroot resistant canola cultivars in the marketplace are truly worrisome, highlighting the dire need for new CR sources as well as strategies. Initial screening of our CR sources indicated that most of the single CR genes are unable to control many of the new pathotype variants identified (2B, 3A, 5X etc.). There might be opportunities to use a multi-genic approach for potentially broader spectrum and greater durability of resistance. Pyramiding different CR genes into canola lines is a potential deployment strategy to explore, but the advantages/disadvantages with this multi-genic approach are largely unknown, especially in terms of the resistance efficacy and longevity in clubroot management.

The main purpose of this project was to assess the efficacy and durability of canola lines carrying single and multiple CR genes under a simulated intensive canola-growing condition, with selected canola lines exposed repeatedly to the same population of a *P. brassicae* (Pb) pathotype. All the genotypes selected are resistant to the predominant pathotype 3H, but the efficacy against some of the newly identified pathotypes had not been determined. Additionally, resistance durability under heavy (Alberta) and lighter (Saskatchewan and Manitoba) infestation situations would also be studied against pathotype 3H to better understand the risk of resistance erosion in recommending a CR canola cultivar in regions with a lower clubroot pathogen inoculum load on the prairies. The mechanisms of resistance associated with CR genes were examined using transcriptome analysis to differentiate the efficacy of CR gene(s) behind the multi-genic approach. The information will provide insights into the modes of action for clubroot resistance and encourage breeders to considered effective CR gene combinations in a multi-genic resistance strategy for clubroot management.

Materials and Methods

Clubroot resistant *B. napus* lines

Initially 20 canola (*B. napus*)-quality inbred and hybrid lines carrying single, double and triple CR genes (**Table 1**) were produced, through reciprocal crosses, in collaboration with Nutrien Ag Solutions. Three of the lines carried only one CR gene (*RRcr1, CRM* or *CRB*), 14 carried two CR genes (double-CR lines, in several breeding background), and the rest carried three CR genes (triple-CR lines, *Rcr1//CRM/CRB* in different breeding background). The CR gene *Rcr1* and *CRM* are within a close range on chromosome A3 of brassica A genome, while *CRB* has been located on chromosome A8. All lines showed resistance to 'old' Pb pathotype (2, 3, 5, 6, 8). The pathotype 3 (now 3H) has been the predominant race in western Canada.

First, these canola lines were assessed for resistance against three field populations of newly identified pathotype 5X, i.e. L-G1, L-G2 and L-G3 (Strelkov et al. 2018) using the inoculum from Dr. Strelkov's lab at UoA. A Pb resting-spore suspension at 1×10^7 spores/ml was applied to the soil above the seed at 3.5 ml per pot, and another 3.5 ml around base of canola seedling 7 d after seeding. Clubroot symptoms were assessed at about 35 days post inoculation (dpi) for L-G2 and L-G3, but at 42 dpi for L-G1 due to slightly less aggressiveness of this population. Symptoms were photographed and disease severity index (DSI) calculated for each line using the following formula. Westar and 45H29 (resistance to old pathotypes), both susceptible to the 5X, were included as controls. The experiment was repeated once.

$$DSI = \frac{\Sigma \text{ (rating class) X (number of plants in the rating class)}}{\text{Total number of plants X 3}} \text{ X } 100$$

Table 1 Canola inbred/hybrid lines carrying single, double and triple CR genes tested in the study.

Entry	Coding	CR gene involved	# CR genes
1	PS-SY 15-3940	*CRM/CRB*	1
2	PS-ARJ 15-3941	*CRM/CRB*	1
3	PS-SY 15-3944	*CRM/CRB*	1
4	PS-ARJ 15-3945	*CRM/CRB*	1
5	PS-SY 15-3948	*CRM/CRB*	1
6	PS-ARJ 15-3949	*CRM/CRB*	1
7	PS-SY 15-3952	*CRM/CRB*	1
8	PS-ARJ 15-3953	*CRM/CRB*	1
9	PS-FBZ 15-4010	*Rcr1//CRM*	1
10	PS-FBZ 15-3967	*Rcr1//CRM/CRB*	3
11	PS-FCA 15-3968	*Rcr1//CRM/CRB*	3
12	PS-FCC 15-3969	*Rcr1//CRM/CRB*	3
13	PS-FCA 15-3978	*Rcr1*	1
14	PS-ARK 14-3562	*CRB/Rcr1*	2
15	PS-ARK 13-2998	*CRB/CRM*	2
16	PS-SY 12-1581	*CRM*	1
17	PS-FBT 15-3987	*CRM//Rcr1*	2
18	PS-FBU 15-3970	*CRM//CRM/CRB*	2
19	PS-FCE 15-4004	*CRM/CRB//CRM*	2
20	SC15NB3-01	*CRB*	1

Transcriptome analysis of double CR genes for mechanisms against pathotype 5X

Canola lines, carrying the CR gene *CRB* on chromosome A08 (Line 20), *CRM* ("Mendel" derived) on chromosome A03 (Line 6), and both *CRB* and *CRB* (Line 15), were used for RNA-seq analysis to decipher molecular mechanisms of the double-CR against Pb pathotype 5X (L-G2), to which both the lines 15 and 20 showed an intermediate level of resistance; the purpose was to determine whether the double CR genes would provide additional modes of action over the single *CRB*. Inoculated roots were sampled at 14 dpi. All samples from the same genotype in a replicate (pot) were pooled, flash frozen in liquid nitrogen and ground with a mortar and pestle. RNA was extracted using the QIAGEN RNeasy Plant mini kit on a QIAcube with a DNase I on-column digestion. The concentration and integrity of resulting RNA was assessed using Nanodrop and Experion (Bio-Rad Canada, Mississauga, ON) automated electrophoresis, respectively.

Sequencing cDNA libraries were constructed using approximately 1 µg of total RNA per pooled sample with a TruSeq™ RNA library Prep Kit (Illumina, San Diego, USA) following manufacturer's protocol. The libraries were sequenced on the Illumina HiSeq 2500 at McGill University and Genome Quebec Innovation Center (Montreal, QC). Raw reads were filtered by removing adaptor sequences, as well as unreliable low-quality reads (quality score <0.05; ambiguous nucleotides >2). The trimmed clean reads for each library were mapped to the reference genome of *B. napus* (http://www.genoscope.cns.fr/brassicanapus/) using CLC genomics workbench (Qiagen, Aarhus, Denmark). The transcript abundance was calculated using the Reads Per Kilobase of exon model per Million mapped reads (RPKM) method (Mortazavi et al., 2008). The false discovery rate (FDR) method (Benjamini and Yekutieli, 2001) was used to determine the threshold of *P* values for multiple tests. Genes were deemed to be differentially expressed when their absolute value of fold change was ≥ 2 with FDR ≤ 0.01.

To identify the putative function of differentially expressed genes (DEGs), a sequence similarity search was carried out against the non-redundant protein database of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) using BLASTX algorithm with an E-value threshold of 1e-5. The Blast2Go-pro suite (Conesa and Gotz, 2008) was used for functional classification and enrichment analysis based on the gene ontology (GO) terms.

Durability of selected *B. napus* lines against pathotype 5X

Two of the 20 lines (14, 15) which showed moderate resistance to each of the three pathotype 5X populations were selected for resistance durability studies against the field population of L-G3 or 3H under controlled-environment conditions mimicking repeated exposure to a Pb population. In the case of L-G3 (5X), the main purpose was to assess the longevity of the intermediate resistance provided by two CR genes on chromosomes A3 and A8 (A3/A8) under high pathogen inoculum pressure (10^7 spores/g soil). For the 3H, different levels of infestation are present on the prairies, ranging from trace to extremely high inoculum loads up to 10^7 spore/g soil in some patches. We intended to investigate how the low vs. high inoculum levels would affect the durability of resistance associated with single and double CR genes.

Against L-G3, additional 5 lines were used as controls, besides the moderately resistant lines 14 and 15, including line 6 (*CRM/CRB*), 12 (*Rcr1//CRM/CRB*), 13 (*Rcr1*), 16 (*CRM*) and 20 (*CRB*), which were susceptible to this 5X population (**Fig 2**). Test lines were grown in Sunshine #3 soil in 8-in diameter plastic pots, by planting 23 seeds of each line plus two Westar seeds as additional controls to confirm successful inoculation for each pot. Three pots were used each test line as replicates. Seeded pots were placed in a growth room set at 22/16°C (day/night) with a 16 h photoperiod. The 5X inoculum was increased by inoculating the cultivar 45H29, resistant to 3H and other "old" pathotypes found in Canada but susceptible to the 5X. Galls were dried at room temperature for a day before being buried in damp growth media for 3 weeks to allow the maturation of resting spores. Mature galls were stored at -20°C until use.

To simulate field infestation, Pb resting spores were premixed in growth media to the concentration of about 1×10^7 spores/g (microscopic enumeration) for the first round of inoculation, referred as 1st generational cycle of exposure. Each line was planted directly into the infested growth media and clubroot symptoms assessed at 35 dpi. Disease severity was rated using a 0-3 scale and fresh clubs from each generational cycle

were dried for a day before being recycled in the media used for the subsequent round of testing. At the beginning of each generational cycle, including the 1st one, approximately 20cc of soil samples were collected and subjected to quantification of pathogen inoculum using quantitative PCR (qPCR). **Fig 1** shows the process of study. The whole process was repeated once, with all materials prepared independently. The experiment lasted about 18 months.

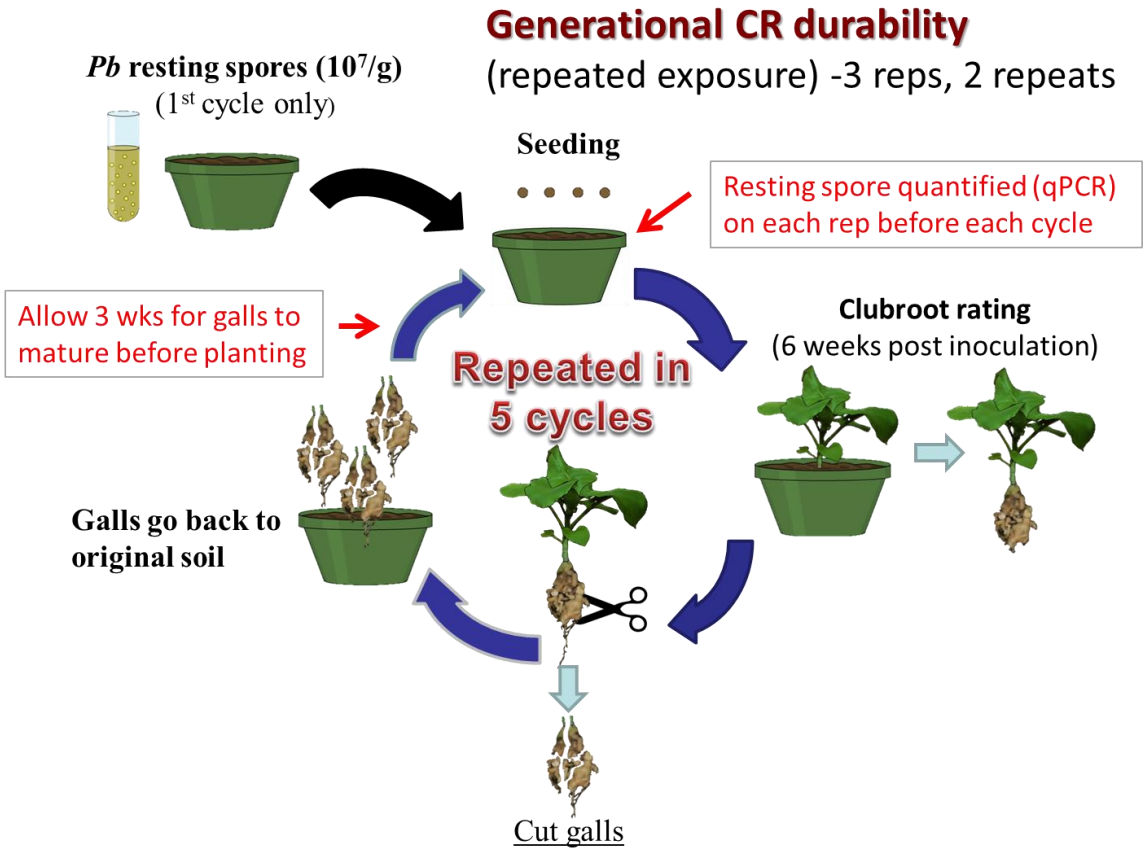

Fig 1 Resistance durability experiment: Setup and process.

Quantification of resting spores in growth media using qPCR: Samples of 0.1 g of growth media infested by Pb resting spores were added to PowerBead tubes, homogenized three times, and DNA extracted using the PowerSoil DNA Isolation Kit (Qiagen Canada, Montreal, QC) following the manufacturer's instructions. qPCR protocol was run following Rennie et al. (2011); each 0.1 g growth-media sample was extracted for DNA using a bead ruptor (Omni Int. Kennesaw, GA), and each DNA sample eluted in 100 µl Tris buffer and stored at -20°C until use. The number of resting spores was quantified using the same marker reported by Cao et al. (2007) in a ViiA 7 analyzer (Thermo Fisher Scientific Canada, Toronto, ON). Extracted DNA samples were diluted twice in 200 µL ddH2O. The PCR reaction was performed in a 20 µL reaction volume consisting of 5 µL template DNA, 10 µL SYBR green, 2 µL primers and 3 µL ddH2O. There were 2 technical replications for each biological replicate (a total of 6 per treatment).

Durability of selected *B. napus* lines against pathotype 3H

A similar protocol, as shown in **Fig 1**, was used to study the resistance durability against pathotype 3H, with some modifications. 1) Two levels of inoculum were used in the 1st generational cycle; about 10^7 spores/g soil (high) and 10^4 spores/g (low), respectively. These different levels of inoculum would represent some of the heavy infestations observed in Alberta and light infestations common in Saskatchewan and Manitoba. 2) Only 3 of the lines were used in this study, i.e. the line 13 (*Rcr1*), 14 (*CRB/Rcr1*) and 20 (*CRB*), because most of the lines developed are resistant to the 3H. 3) Each pot contained 9 plants of a test line, plus 3 plants of each Westar and 45H29 to confirm successful inoculation with each pot and detect the sign of pathogen population changes towards virulence against the "Mendel" resistance. 4) At the beginning of each generational cycle, 20cc of soil samples were collected but the Pb inoculum quantification used droplet-digital PCR (ddPCR) on A QX200TM System (Bio-Rad, Mississauga, ON), instead of qPCR. The experiment consisted of 4 generational cycles and was repeated once. The whole process of experiment last about 15 months.

The ddPCR reaction mix containing probe supermix, primers, probe and sample cDNA was partitioned into aqueous droplets in oil via a droplet generator. The droplets were transferred to a 96-well PCR plate and a thermocycling process was carried out on a conventional thermal cycler. The PCR plate was then transferred to the droplet reader for data analysis using QuantaSoftTM Software. A threshold of 2000 was set to differentiate positive droplets relative to the background fluorescence. Compare to qPCR, ddPCR can be less affected by potential inhibitors in soil samples, providing more accurate estimation of Pb inoculum concentration.

Data analysis

All experiments were repeated and data were examined for normal distribution based on the Shapiro-Wilk Test. Disease severity index (DSI) and Pb resting spore enumeration data were Log transferred for normal distribution. The homogeneity of variances from repeated trials were confirmed with the Bartlett's Test before being pooled for analysis. DSI or resting spore concentration estimated using PCR were subjected to analysis of variance using Proc ANOVA in SAS (SAS Institute Inc. Cary, NC), and the means were separated using LSD ($P = 0.05$) when ANOVA was significant ($P \leq 0.05$).

Results and Discussion

Resistance of developed canola lines to different populations of pathotype 5X

Among the 20 canola inbred/hybrid lines tested, two of the double CR-gene lines (14, 15) and one single gene line (20) showed a moderate level of resistance to L-G1 and L-G2, with DSI < 40% (**Fig 2**; **Fig 3**; **Fig 4**). However, only the lines 14 and 15 were moderately resistant to LG-3, and the rest were susceptible, with DSI ranging from 50% to 100% (**Fig 2**; **Fig5**). The only difference between the lines 14 and 15 from the other double CR-gene lines (susceptible) is that they were produced with the *CRB*-carrying line as a female crossed with a line carrying *Rcr1*

or *CRM* as a male, so it appears that something might have been lost during crosses when a *CRB*-carrying line was used as a male. It is possible that *CRB* is not a single gene; on chromosome A08 of *B. rapa* close to the locus of *CRB* (Hasan and Rahman 2016), at least two CR loci (*Crr1a*, *Crr1b*) have been identified previously (Suwabe et al. 2003; 2006). *Crr1a* has been cloned (Hatakeyama et al 2013) while *Crr1b* still remains a bit elusive. The variations of resistance may be depend on if both CR loci on A8 were retained. Even with both CR loci on A08 of *B. rapa*, a line (20, for example) may confer moderate resistance to L-G1 or L-G2, but not to L-G3. The resistance to the latter may require the presence of both *CRB* (A8) and *Rcr1* or *CRM* (A03). This result suggests that *CRB* plays an interesting role in regulating resistance to pathotype 5X, interacting with *Rcr1* or *CRM* for resistance against each of the three 5X populations.

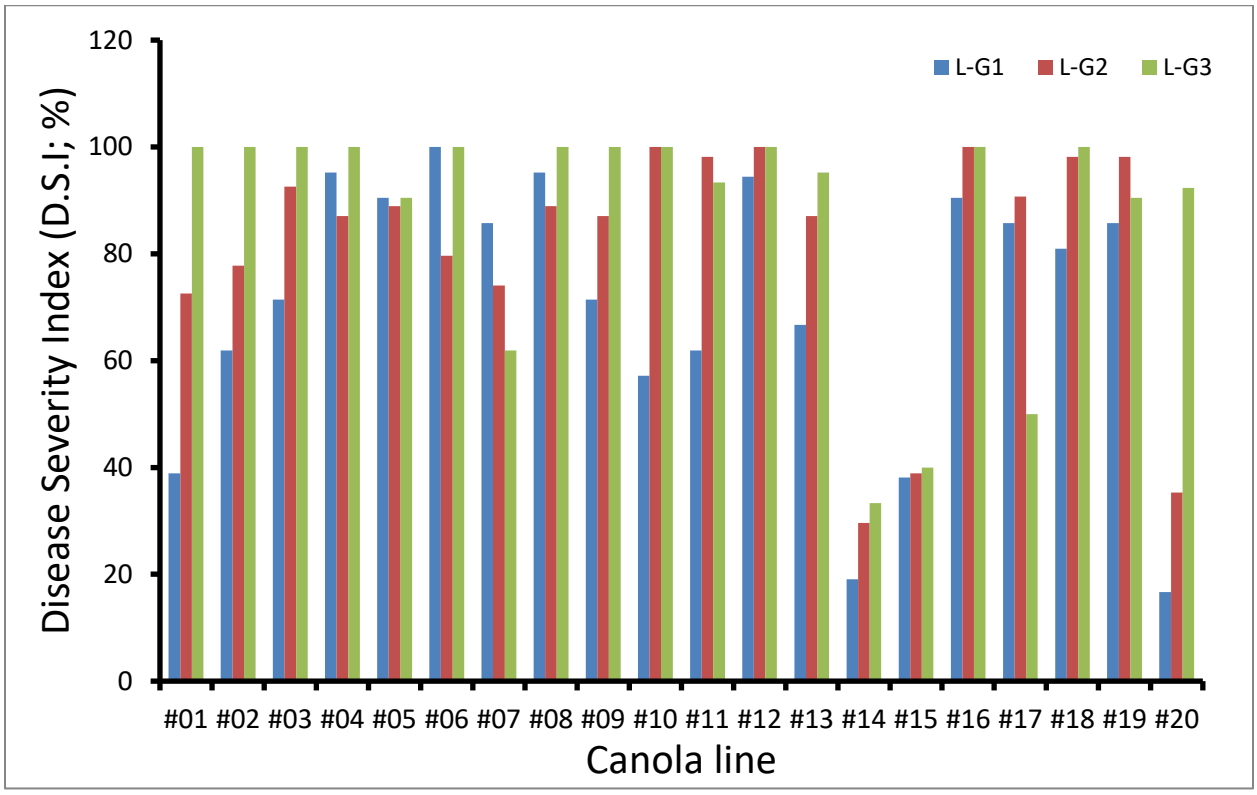

Fig 2 Disease Severity Index (DSI) from 20 canola lines carrying different number of CR genes in response to inoculation by L-G1, L-G2 and L-G3 of pathotype 5x, respectively. The DSI on Westar (susceptible control) and 45H29 (Mendel resistance) were 100%.

Fig 3 Representative clubroot symptoms on 20 canola lines carrying sing-, double- and triple-CR genes against the L-G1 population of pathotype 5X. The stars indicate partial resistance observed.

Fig 4 Representative clubroot symptoms on 20 canola lines carrying sing-, double- and triple-CR genes against the L-G2 population of pathotype 5X. The star indicate partial resistance.

Fig 5 Representative clubroot symptoms on 20 canola lines carrying sing-, double- and triple-CR genes against the L-G3 population of pathotype 5X. The stars indicate partial resistance.

Transcriptome analysis of double-CR lines against pathotype 5X (L-G2)

Both lines 15 (*CRB/CRM*) and 20 (*CRB*) showed a moderate level of resistance to this 5X population, whereas the line 16 carrying *CRM* alone appeared susceptible. RNA-seq analysis showed that the

inoculation induced the most number of differentially expressed genes (DEGs) in Line 15, relative to non-inoculated (mock –M) control (**Fig 6**). DEGs were fewer in the comparison for line 20 and fewest in line 16. DEG annotation showed that many pathways were activated in the partially resistant Lines 15 and 20 (**Fig 7**), but much fewer in the susceptible line 16 relative to the mock. Enrichment analysis showed that line 15 shared more DEGs with line 20 than with line 16 (**Fig 8**). Many genes involved in pathogen associated pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) were activated in inoculated line 15 and 20, but not 16 (**Table 2**).

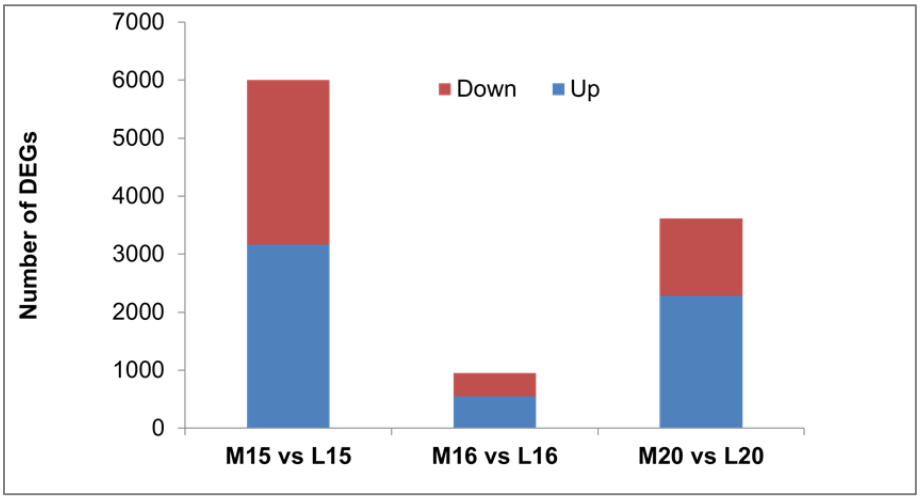

Fig 6 Up- (red) and down- (blue) regulated DEGs based on the absolute fold value change >4 and *P*-value ≤ 0.01 between non-inoculated (mock -M) and *P. brassicae* inoculated root samples.

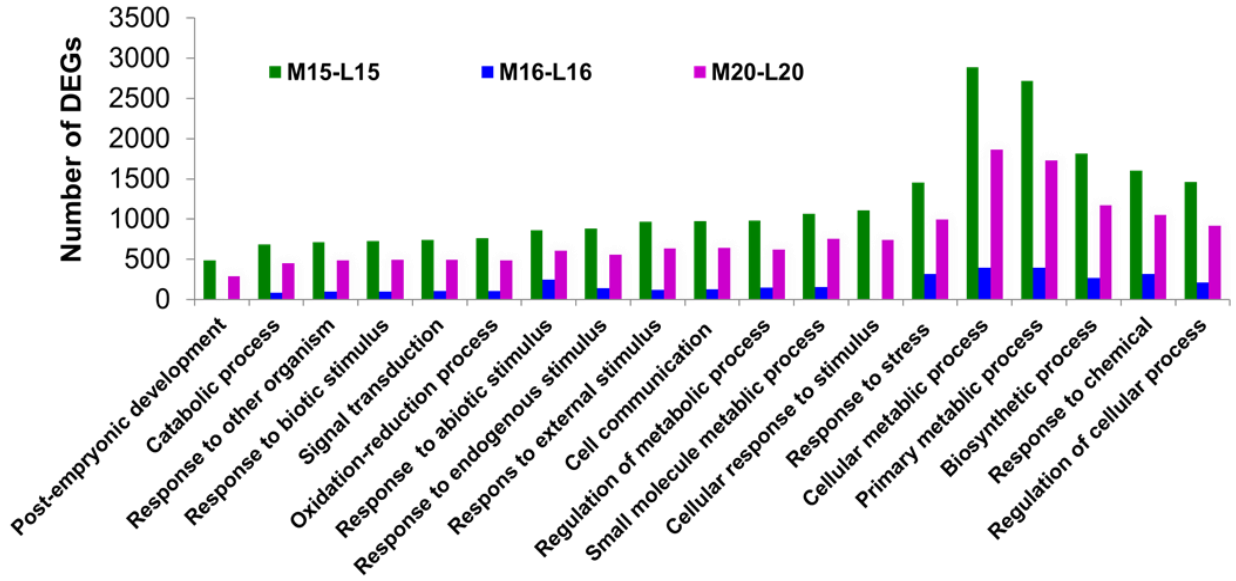

Fig 7 Assignment of DEGs to selected biological processes based on gene ontology (GO) terms.

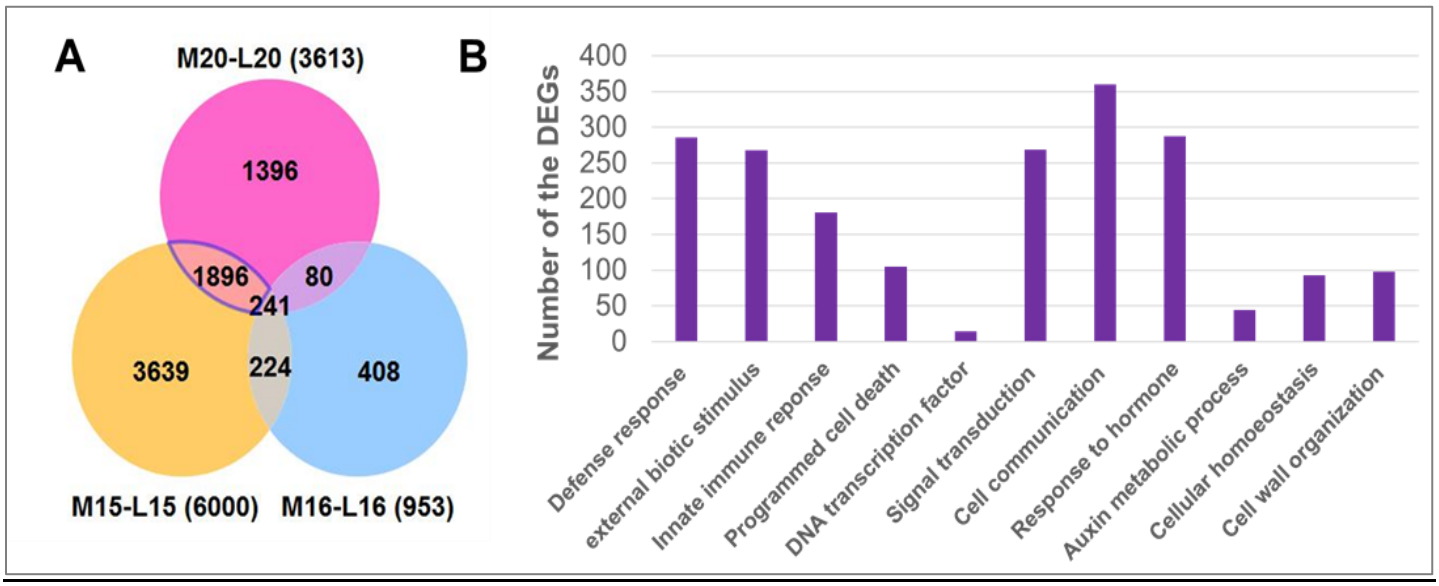

Fig 8 Enrichment analysis: Distribution and sharing of DEGs among three canola lines (A), and disease-resistance pathways shared between the lines 15 and 20, but not with line 16.

Table 2 Fold changes of DEGs involved in PTI and ETI caused by the inoculation with pathotype 5X (L-G2).

Pathway	Differentially expressed genes	M15-L15	M16-L16	M20-L20
PTI	Receptor-like protein kinase 5	34.38	2.14	6.94
	Receptor-like protein 12	760.20	2.09	36.58
	Cysteine-rich receptor-like protein kinase 11	18.50	1.56	29.71
	Cysteine-rich receptor-like protein kinase 5	18.75	3.29	4.83
	Wrky transcription factor 70	177.15	2.74	30.55
	Wrky transcription factor 51 isoform x1	42.99	1.73	7.54
	Wrky transcription factor 38	20.30	3.14	6.50
ETI	Probable disease resistance protein1	85.56	1.55	5.92
	Disease resistance protein1	16.26	1.18	6.25
	Probable disease resistance protein2	23.00	3.74	5.06
	Disease resistance protein2	22.15	1.60	6.27
	Enhanced disease susceptibility 1	44.86	1.37	13.06
	Isochorismate synthase	19.34	2.68	20.62

The lines 15 with two CR genes (*CRB/CRM*) and line 20 with a single CR gene (*CRB*) both conferred intermediate resistance against L-G2 of the 5X, and the resistance may be resulted from the activation of genes involved in both PTI and ETI pathways, relative to those in line 16 (**Table 2**). It is noteworthy that most of the DEGs involved in PTI and ETI showed higher transcriptional levels in the double CR-gene line than in line 20 (*CRB*); this may indicate that the two CR genes together trigger stronger defense responses than the single gene *CRB* alone, hence implying the advantage of stacking those CR genes. Further studies are required to confirm the contribution of higher transcriptional expression of these PTI- and ETI-related genes to resistance performance and durability.

Resistance durability of the lines 14 and 15 against pathotype 5X (L-G3)

The two double-CR lines, 14 (*CRB/CRM*) and 15 (*CRB/Rcr1*), held the moderate levels of resistance to L-G3 through five generational cycles of exposure, with DSI generally < 30% (**Fig 9**; **10**; **11**; **12**), despite the fact that all root galls (small though) were recycled back into the growth media at the end of each generation. The other five lines carrying single, double or triple CR genes in different breeding background were susceptible, with DSI >50%. The two trial repetitions showed a similar trend, and the results presented in graphs combined the data from the two repetitions.

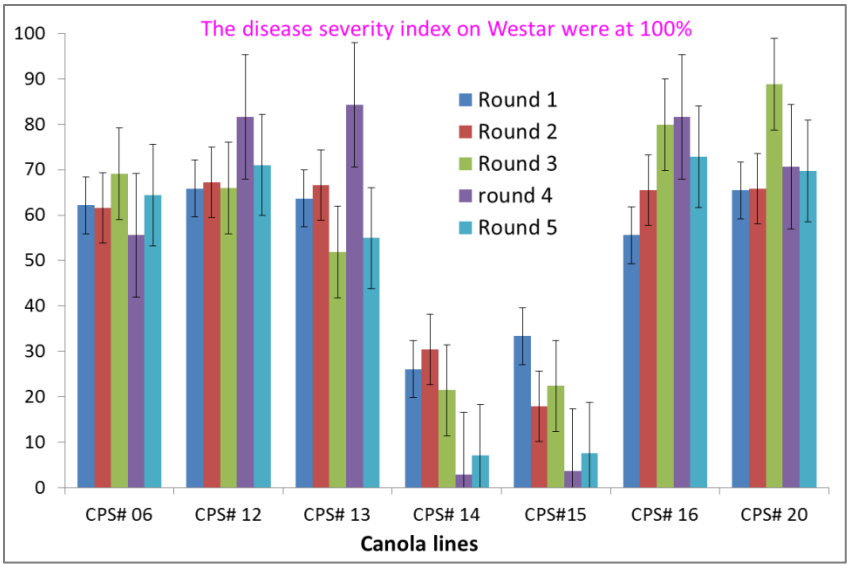

Fig 9 Disease severity index (%) on canola lines carrying single, double or triple CR genes in repeated exposure (5 cycles) to a field population of *Plasmodiophora brassicae* pathotype 5X (L-G3) based on two repeated trials. CPS# = line#

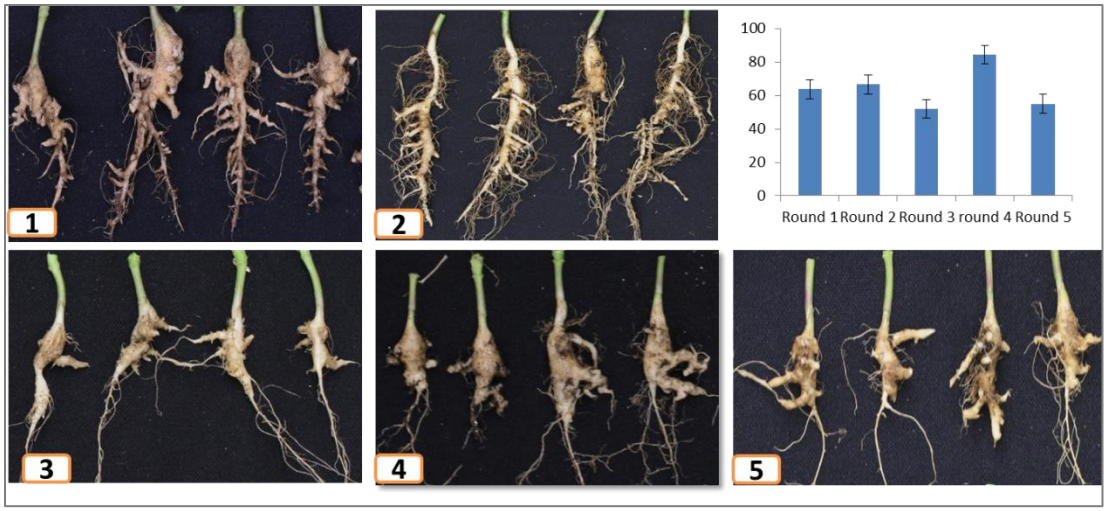

Fig 10 Clubroot symptoms on the canola line 13 (*Rcr1*) inoculated with pathotype 5X of *Plasmodiophora brassicae* (L-G3) in five generational rounds (cycles) of exposure. The bar graph shows the average disease severity index (%) in each of the cycles.

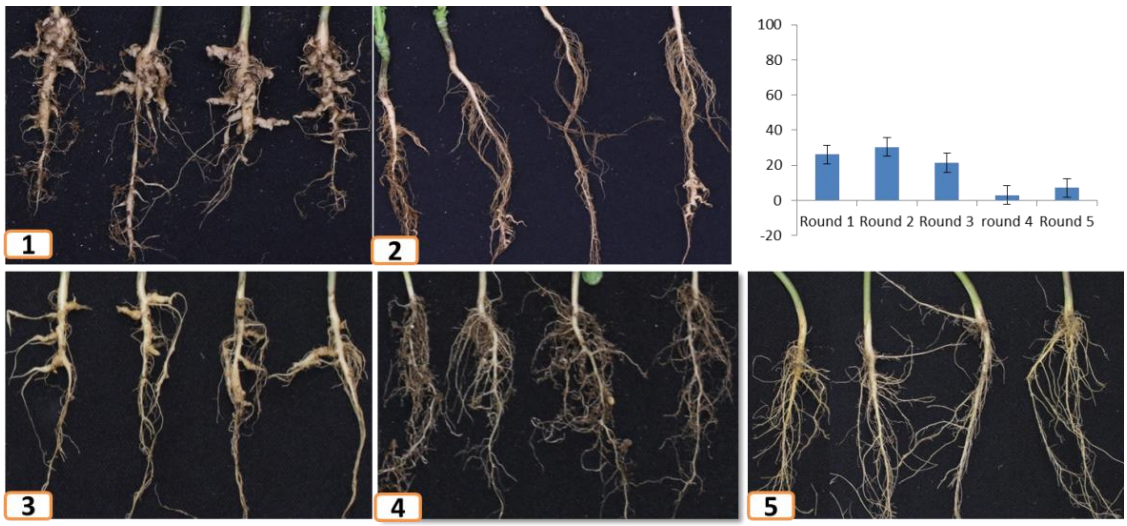

Fig 11 Clubroot symptoms on the canola line 14 (*CRB/Rcr1*) inoculated with pathotype 5X of *Plasmodiophora brassicae* (L-G3) in five generational rounds (cycles) of exposure. The bar graph shows the average disease severity index (%) in each of the cycles

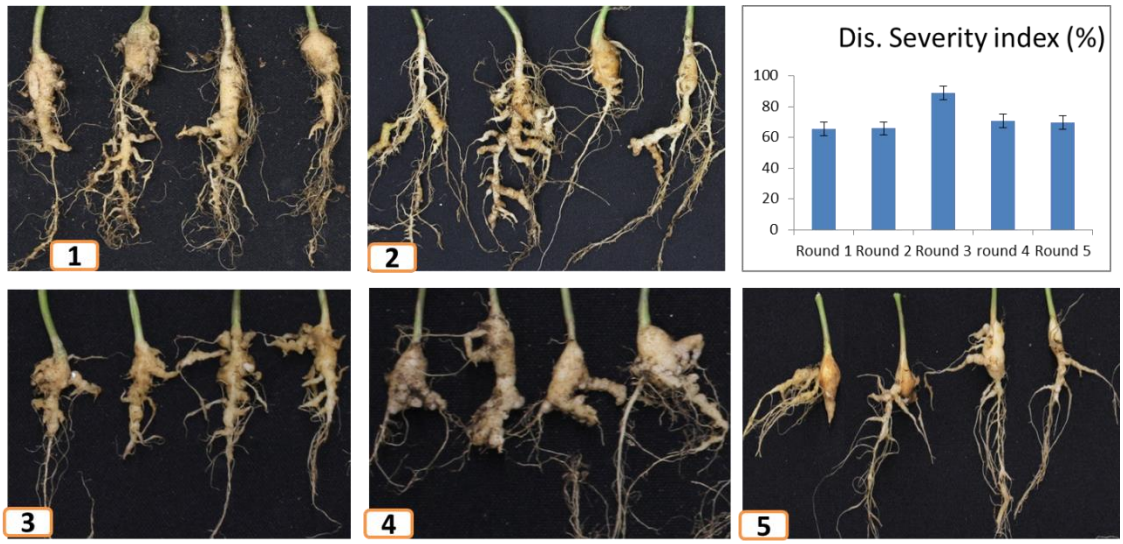

Fig 12 Clubroot symptoms on the canola line 20 (*CRB*) inoculated with pathotype 5X of *Plasmodiophora brassicae* (L-G3) in five generational rounds (cycles) of exposure. The bar graph shows the average disease severity index (%) in each of the cycles

Based on qPCR results, the resting-spore concentration added to the growth media of different canola lines was similar (~1E+07/g media) at the start of experiment (**Fig 13**). The inoculum level changed slightly from generation to generation; there was a slight decrease in resting spore concentration for the moderately resistant lines 14 (*CRB*/*CRM*) (**Fig 13**) and 15 (*CRB/Rcr1*, data not shown), which coincided with a decline in the disease severity index (DSI).

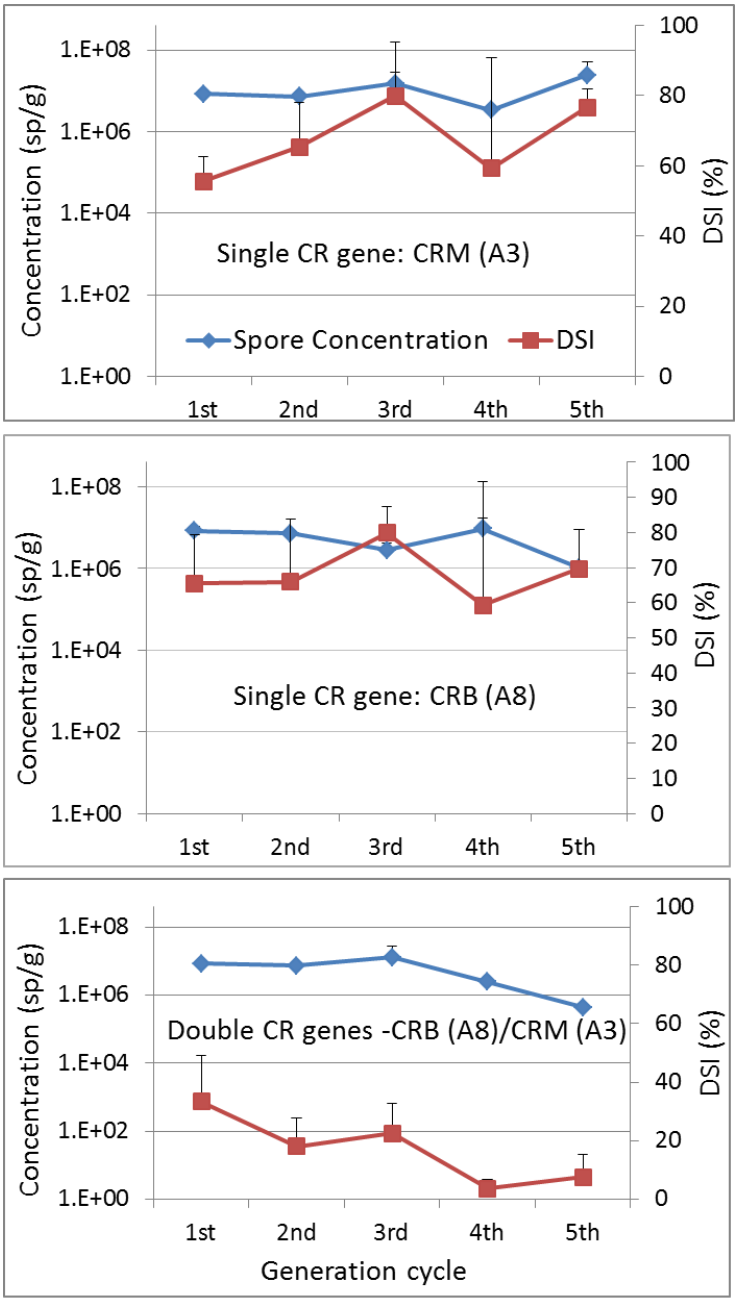

Fig 13 Comparison of single- (*CRM* or *CRB*) and double-CR (*CRB/CRM*) lines for resting-spore concentration (spore/g media) and disease severity index (DSI) in five generational cycles of exposure to pathotype 5X (L-G3).

<u>Durability of resistance against pathotype 3H:</u>

Since pathotype 3H is the most common Pb race on the prairies, it remains the primary target for genetic resistance development. Each of the 20 canola lines carries at least one CR gene which confers resistance to pathotype 3H. Therefore, all these lines were expected to be resistant to this pathotype initially. Under the low initial inoculum level (10^4 spores/g), susceptible Westar showed relatively severe DSI right from the beginning and the disease level was maintained in subsequent generational cycles (**Fig 14**). No clubroot was observed on any of the lines at the end

of first two cycles, but a low level of disease was seen on line 20 (*CRB*) in cycle 3 and 4, while the other two lines (*Rcr1*; *CRB/Rcr1*) were generally clubroot free.

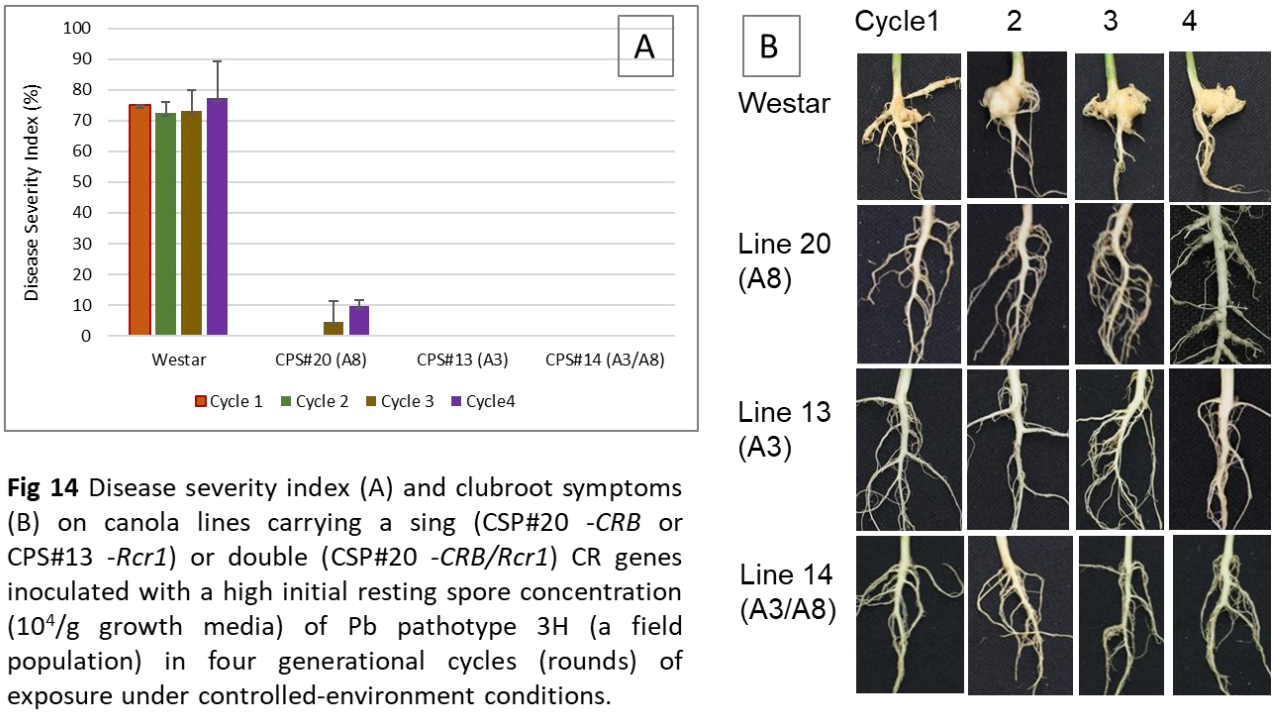

Fig 14 Disease severity index (A) and clubroot symptoms (B) on canola lines carrying a sing (CSP#20 -*CRB* or CPS#13 -*Rcr1*) or double (CSP#20 -*CRB/Rcr1*) CR genes inoculated with a high initial resting spore concentration (10^4/g growth media) of Pb pathotype 3H (a field population) in four generational cycles (rounds) of exposure under controlled-environment conditions.

Under the high initial inoculum level (10^7 spores/g), however, the susceptible control showed slightly higher DSI (**Fig 15**), relative to the low-inoculum level (**Fig 14**), but the disease level was not substantially different among the five generational cycles. A trace-level clubroot was observed on line 20 (*CRB*) in cycle 1, and there seemed a trend of gradual DSI increase as the generational cycle progressed (**Fig !5A**). No clubroot was observed on line 13 (*Rcr1*) in cycle 1 and 2, but a trace level of disease was seen in cycle 3 and 4. In comparison, the double CR-gene line 14 (*Rcr1/CRB*) was generally free of clubroot until the end of cycle 4 where only one plant showed slight bulging at the base of a branch root (**Fig 15B**).

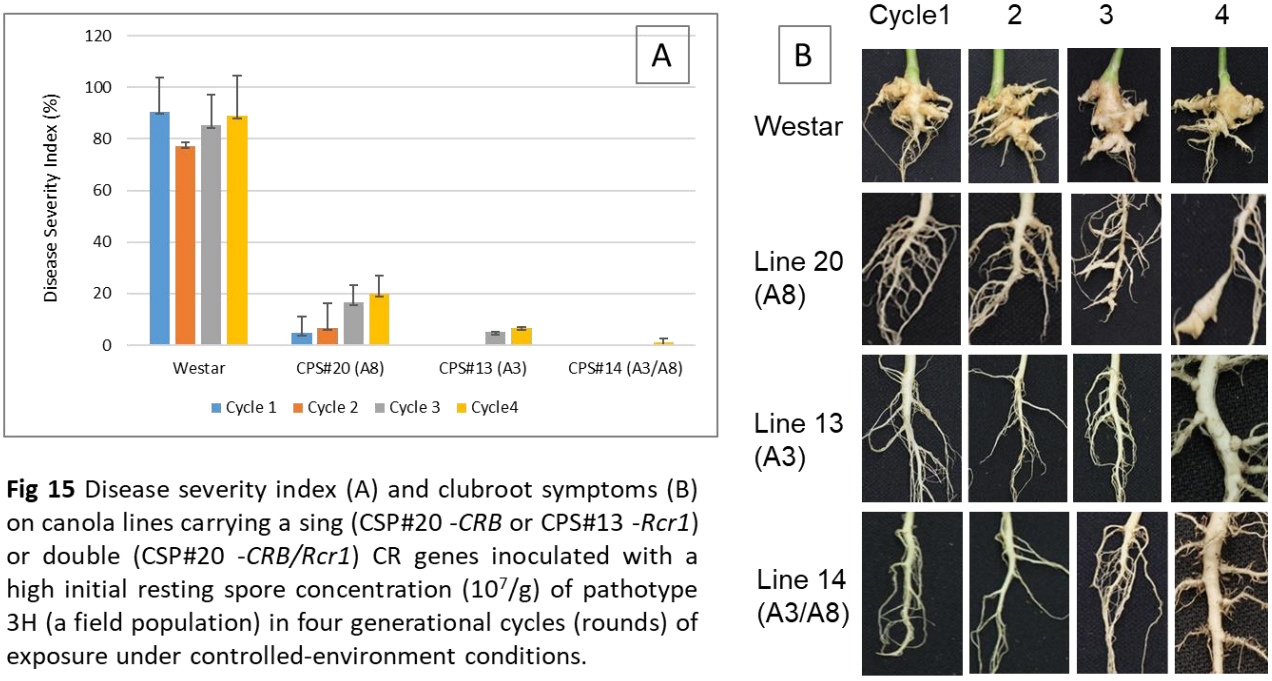

Fig 15 Disease severity index (A) and clubroot symptoms (B) on canola lines carrying a sing (CSP#20 -*CRB* or CPS#13 -*Rcr1*) or double (CSP#20 -*CRB/Rcr1*) CR genes inoculated with a high initial resting spore concentration (10^7/g) of pathotype 3H (a field population) in four generational cycles (rounds) of exposure under controlled-environment conditions.

ddPCR results showed that there was generally a decrease in Pb resting spores in the growth media infested with either low (10^4 /g) or high (10^7/g) levels of inoculum in the cycle 1 (**Fig 16**). It appears that the decline in inoculum through the generational cycle is resulted from the resistance of canola lines; no or only a few small galls were formed on the roots line 14 or 15, which would not replenish the inoculum reduced in the growth media. Despite the general decrease in Pb inoculum, clubroot symptoms seemed to increase gradually, especially on the line carrying *CRB* only (line 20) under high Pb inoculum pressure. The inoculum of pathotype 3H was from a heavily diseased canola field in Alberta, so it is possible that other pathotypes were present in the inoculum, likely at very low levels. This notion is supported by the trace-level clubroot symptoms on line 20, supposedly resistant to pathotype 3H, in the cycle 1 of exposure under high inoculum pressure (**Fig 15**). While the overall inoculum level declined through generational cycles, it was possible that the proportion of virulent inoculum increased due to the recycling of virulent inoculum in small galls back into the media, which resulted in increased clubroot DSI over the generational cycle (**Fig 14, 15**).

This experiment showed that the identification of a dominant pathotype (3H, for example) in a field Pb population would not necessarily exclude the presence of other pathotypes at low levels. When one or more of these low-level pathotypes are virulent to the resistant cultivar, slight infection may occur and light clubroot symptoms may be present and progressively increase if the same CR gene(s) are used repeatedly. While canola lines carrying the CR genes *Rcr1* or *CRB* are individually resistant to pathotype 3H, lines with *Rcr1* appeared more efficacious in terms of resistance resilience under both low and high levels of Pb inoculum, with slightly delayed/lowered presence of clubroot infection (**Fig 14, 15**). Higher inoculum load accelerates the resistance erosion, especially for canola varieties carrying a single CR gene. This would call for extended crop rotation (Peng et al. 2015, Gossen et al. 2017) to reduce the load of Pb resting spores in heavily infested fields to assist the performance and durability of CR genes. Canola line carrying both *Rcr1* and *CRB* showed slightly better

resistance durability, relative to either single CR gene alone, against virulent pathotypes at low inoculum levels. This advantage, however, may need to be interpreted with caution because one of the line 14 plants did show the sign of infection at the end of 4th cycle under high initial inoculum load (**Fig 15**).

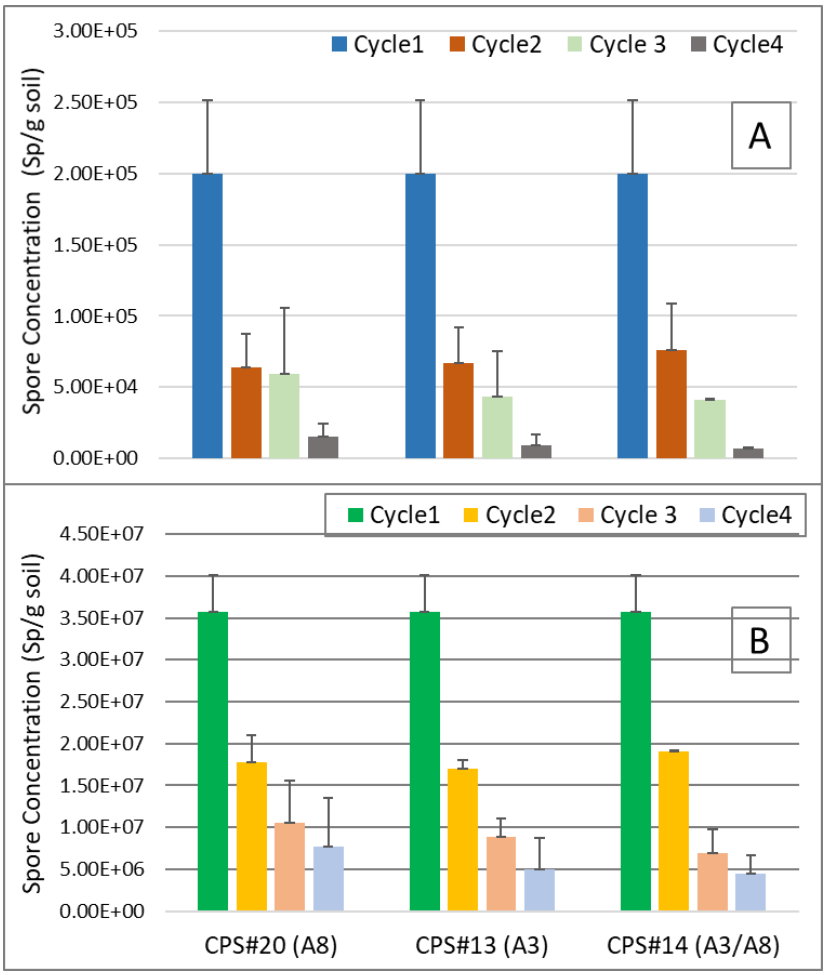

Fig 16 Comparison of single- (*Rcr1* or *CRB*) and double (*CRB*/*Rcr1*) CR-gene lines for resting-spore concentration in growth media (spore/g) in four generational cycles of exposure to pathotype 3H of *Plasmodiophora brassicae*.

Concluding remarks

This is a unique study investigating CR durability using canola lines/varieties carrying single and double CR genes against "new" and "old" Pb pathotypes. In the experiment, clubroot galls were recycled back into the soil to mimic the field conditions where diseased roots remain in the field at the end of each crop season, although the duration of generational cycle was much shorter in the current study, with 8 weeks for clubroot to develop after seeding and 3 weeks for recycled galls to disintegrate and release the inoculum in the soil. Time and space were the key limitations to extend the generational cycles. Nevertheless, the study provided valuable information for

development of new management strategies against clubroot on the prairies. First, CR genes on chromosome A8 (*CRB*) are effective to old pathotype 3H but susceptible to L-G3 of the new pathotype 5X. When combining *CRB* with one of the CR genes on chromosome A3 (*Rcr1* or *CRM*), however, moderate resistance was achieved against all 5X populations, as well as the old pathotypes 2, 3, 5, 6 and 8. This indicates that the range of resistance can be increased by stacking two CR genes with different modes of action. Many genes involved in PTI and ETI pathways were more strongly activated in lines carrying these two CR genes, relative to those controlled by either the single CR gene alone, in response to 5X infection.

It is a bit surprising to see the durability of intermediate resistance against the 5X, especially in light of an earlier study against pathotype 3 (LeBoldus et al. 2012). In the current study, the efficacy was held through five generational cycles of exposure, with DSI generally < 30%, despite the fact that a high level of Pb inoculum was applied at the beginning of the experiment and all root galls were recycled back into the soil at the end of each generational cycle. This highlights the value to use stacked CR genes of different modes of action for resistance performance and durability. More work is warranted to conduct a study against additional new pathotypes (2B, 3A, for example) to determine the validity of this multi-genic strategy for enhanced resistance efficacy and durability. The data on the old/predominant pathotype 3H showed that field Pb populations are likely mixtures, with some pathotypes present at very low levels. When a CR gene(s) is used repeatedly, selection pressure will be present and consequently, the proportion of virulent pathotypes will increase, so the clubroot severity. Higher inoculum load tends to accelerate the resistance erosion, especially for canola varieties carrying only a single CR gene. This highlights the importance of extended crop rotation (a >2-year break from canola) to reduce the load of resting spores in heavily infested fields to assist in the performance and durability of clubroot resistance.

Tech transfer activities

Peng G. 2017. Using “omics” approaches to decipher mechanisms of clubroot resistance. Int. Clubroot Workshop. April 26-28, 2017. Wuhan, China (Invited Talk).

Song T, Hornaday, K, Tonu N, Yu F, Peng G. 2017. Assessment of strategies to enhance resistance against new clubroot pathogens using current resources. CPS-CSA Joint Annual Mtg, June 18-21, 2017, Winnipeg, MB (Poster)

Peng G, Song T, Chu M, Lahlali R, Tonu N, Hornaday K, Lee J, Bush J, Wen R, Karunakara C, Yu F, 2018. Approaches to improve the understanding of clubroot resistance mechanisms and generational resistance durability. China-Canada Canola Genomics Workshop. Mar 26-28, 2018. Wuhan, China (Invited Talk).

Peng G, Song T, Chu M, Lahlali R, Tonu N, Hornaday K, Lee J, Bush J, Wen R, Karunakara C, Yu F, McGregor L. 2018. Understanding the mechanisms and generational durability of clubroot resistance. Brassicas 2018. July 1-4, 2018. San Malo, France (oral presentation).

Peng G, Song T, Wen R, Tonu N, Yu F. 2018. The effect and durability of incomplete resistance against *Plasmodiophora brassicae* 5X. 2018 Int. Clubroot Workshop. Aug 7-9, 2018, Edmonton, AB (Keynote Speaker).

Wen R, Lee J, Hornaday K, Tonu N, Song T, Yu F, Peng G. 2018. Transcriptome analysis of canola lines carrying single and double clubroot resistance genes against the *Plasmodiophora brassicae* pathotype X-LG2 (5X). *The 5th PPSA-Sask CPS Joint Meeting*, Oct, 15-17, 2018. Lloydminster, AB (Poster).

Peng G. 2019. The mechanism and durability of intermediate resistance to *Plasmodiophora brassicae* pathotype 5X conferred by two resistance genes. 14th Int. Rapeseed Congress, June 16-19, 2019, Berlin Germany (Oral presentation)

Peng G. 2019. Enhancing the durability of clubroot resistance with multiple genes. Saskatchewan Canola Polooza, July 9, 2019 (Speaker).

Acknowledgement

Dr. T Song was the lead scientist in proposing the project. He left the project in 2017 to join the Biotechnology Center of Syngenta AG in Beijing. Drs. N. Tonu and R. Wen carried on with the project to its completion. The quantification of Pb inoculum using qPCR was done at Pest Surveillance Initiative (PSI), Manitoba under a research contract. Later Dr. Wen developed a ddPCR protocol for Pb inoculum quantification with improved accuracy and consistency. Kylie Hornaday and Jillian Lee were Coop students from UBC and UoVic on the project and helped substantially in carrying out a range of project experiments. The research funding provided by WGRF and SaskCanola allowed the hiring of postdocs and students on the project. The financial support is much appreciated.

References

Benjamini Y, Yekutieli D. 2001. The control of the false discovery rate in multiple testing under dependency. Ann Stat. 29: 1165–1188.

Cao, T., Tewari, J., and Strelkov, S. E. 2007. Molecular detection of *Plasmodiophora brassicae*, causal agent of clubroot of crucifers, in plant and soil. Plant Dis. 91: 80-87.

Conesa A, Gotz S. 2008. Blast2GO: A comprehensive suite for functional analysis in plant genomics. Int J Plant Genomics 2008, 619832.

Gossen, B. D., Al-Daoud, F., D., T., D. J., Peng, G., D., P., and McDonald, M. R. 2019. Comparison of Techniques for Estimation of Resting Spores of Plasmodiophora brassicae in Soil. Eur. J. Plant Pathol. 150:341–349.

Hasan MJ, Rahman H. 2016. Genetics and molecular mapping of resistance to *Plasmodiophora brassicae* pathotypes 2, 3, 5, 6, and 8 in rutabaga (*Brassica napus* var. *napobrassica*). Genome 59: 805–815.

Hatakeyama K, Suwabe K, Tomita RN, Kato T, Nunome T, Fukuoka H, Matsumoto S. 2013. Identification and characterization of *Crr1a*, a gene for resistance to clubroot disease (*Plasmodiophora brassicae* Woronin) in *Brassica rapa* L. PLoS One 8: e54745.

LeBoldus JM, Manolii VP, Turkington TK, Strelkov SE. 2012. Adaptation to Brassica host genotypes by a single-spore isolate and population of Plasmodiophora brassicae (clubroot). Plant Dis. 96: 833–838.

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

Peng G, Pageau D, Strelkov SE, Gossen BD, Hwang SF, Lahlali R. 2015. A >2-Year Crop Rotation Reduces Resting Spores of Plasmodiophora brassicae in Soil and the Impact of Clubroot on Canola. Eur. J. Agron. 70: 78-84.

Rennie D. C., Manolii, V. P., Cao, T., Hwang, S. F., Howard, R. J., and Strelkov, S. E. 2011. Direct Evidence of Surface Infestation of Seeds and Tubers by Plasmodiophora brassicae and Quantification of Spore Loads. Plant Pathology 60:811-819

Strelkov SE, Hwang SF, 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: 284–298

Suwabe K, Tsukazaki H, Iketani H, Hatakeyama K, Fujimura M, et al. 2003. Identification of two loci for resistance to clubroot (*Plasmodiophora brassicae* Woronin) in *Brassica rapa* L. Theor Appl Genet 107: 997–1002.

Suwabe K, Tsukazaki H, Iketani H, Hatakeyama K, Kondo M, et al. 2006. Simple sequence repeat-based comparative genomics between *Brassica rapa* and *Arabidopsis thaliana*: the genetic origin of clubroot resistance. Genetics 173: 309–319.