## PROJECT DETAILS

- **Title**: Understanding the mechanisms for race-specific and non-specific resistance for effective use of cultivar resistance against blackleg of canola in Western Canada
- **Funders:** Alberta Canola, SaskCanola, Manitoba Canola Growers, Agriculture and Agri-Food Canada
- **Research program:** Growing Forward 2 and the Canola Agronomic Research Program
- **Principal investigator:** Gary Peng
- **Collaborators/additional investigators:** Fengqun Yu, Linda McGregor, Xunjian Liu, Zhai Chun, Chithra Karunakaran and Rachid Lahlali
- **Year completed:** 2018

**Final report**

**Main objectives of this study:**

1. Determine the type of resistance in representative R-rated common canola cultivars used in western Canada, and characterize their resistance responses to different *Avr* genes;
2. Investigate and develop pathological, molecular and biochemical protocols for efficient and reliable assessment of different types of blackleg resistance, especially the quantitative resistance which does not involve specific R genes.
3. Understand and characterize the mechanisms and efficacy of race-specific and non-specific blackleg resistance based on key pathological, molecular and biochemical analyses during the infection process to elucidate the key mechanisms associated with different types of resistance
4. Assess potential influence of environmental factors, especially those of high temperature/drought, on expression and efficacy of different resistance mechanisms to better understand the role and limitation of different types of resistance under western Canadian conditions.

**There are four key components in this study:**

**1)** Characterizing blackleg resistance associated with common canola cultivars used in western Canada

**2)** Understanding the molecular mechanisms of the resistance gene *Rlm1* based on RNA sequencing

**3)** Molecular mechanisms of quantitative resistance in cotyledons of 74-49 BL revealed by RNA sequencing

**4)** The quantitative resistance against blackleg remains effective under high-temperature conditions.

**1)** Most canola cultivars grown in western Canada with a blackleg-resistant label carry the specific resistance (*R*) gene *Rlm1* and/or *Rlm3*. Recent field monitoring data generally show that the corresponding avirulence genes *AvrLm1* and *AvrLm3* are at very low frequencies or even undetectable in the pathogen population in western Canada, indicating that these *R* genes are no longer effective. Despite this, severe blackleg damage is still uncommon on these resistant cultivars, suggesting additional resistance mechanisms may be present. Three R-rated (blackleg resistant) cultivars carrying *Rlm1* and *Rlm3* were inoculated with virulent isolates of *L. maculans* (without the corresponding *AvrLm1* and *AvrLm3*). "Westar" was used as a susceptible control. The infection in cotyledons and spread of fungal hyphae were assessed using a 0-9 scale and fluorescence microscopy. The amount of *L. maculans* DNA in the petioles and stems linked to hyphal spread and growth was quantified using droplet digital PCR (ddPCR) at 14 days post inoculation (dpi). All inoculated cotyledons showed infection symptoms, but the severity was lower for the R-rated cultivars, with lower plant mortality, relative to Westar (**Fig. 1**). The hyphal spread was more limited in the cotyledon of R-rate cultivars with a virulent isolate of *L. maculans* labelled with green fluorescent protein (**Fig. 2**), and the amount of pathogen DNA was also substantially less in the petioles and stems of R-rated cultivars relative to those in Westar (**Fig. 3**). These results indicate that quantitative resistance (QR) plays a role for these R-rated canola cultivars by reducing the spread of fungal hyphae from infected cotyledons into stems (lower disease incidence) and/or limiting the damage to the stem after the pathogen gets in there (lower disease severity). The QR trait was confirmed with cotyledon inoculation using multiple virulent *L. maculans* isolates separately.

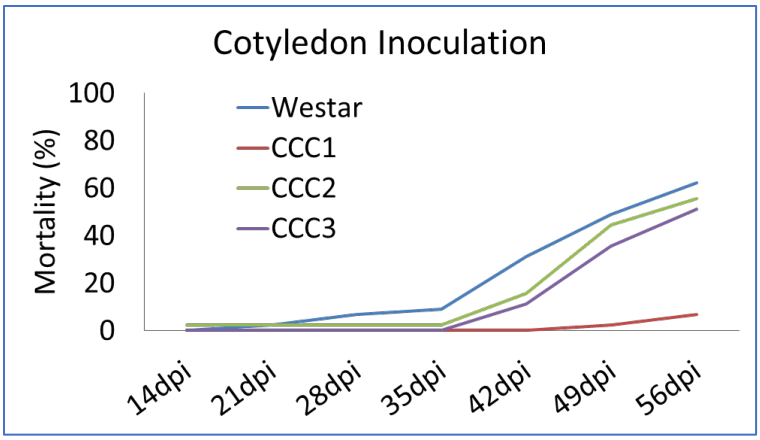


**Fig. 1** Plant mortality of selected Canadian canola cultivars (CCC) originating from the inoculation of cotyledons with the *L. maculans* isolate 12CC09. Westar was a susceptible control.



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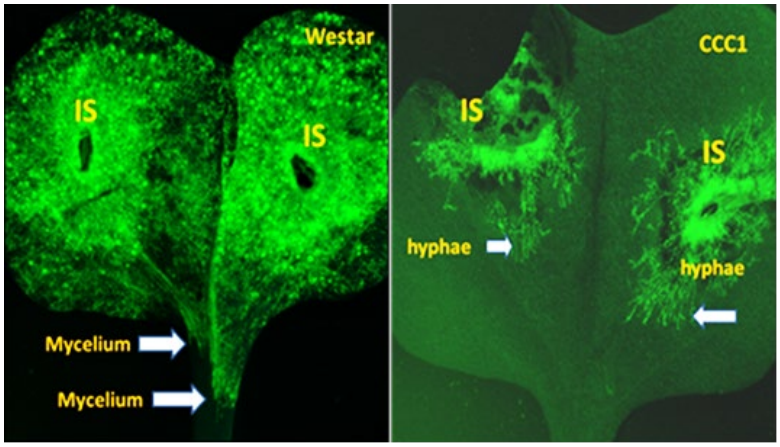


**Fig. 2** The spread of green fluorescent protein (GFP)-labelled *L. maculans* hyphae (white arrows) in cotyledon and petiole tissues from the inoculation site (IS) on Westar and CCC1 at 10 dpi.

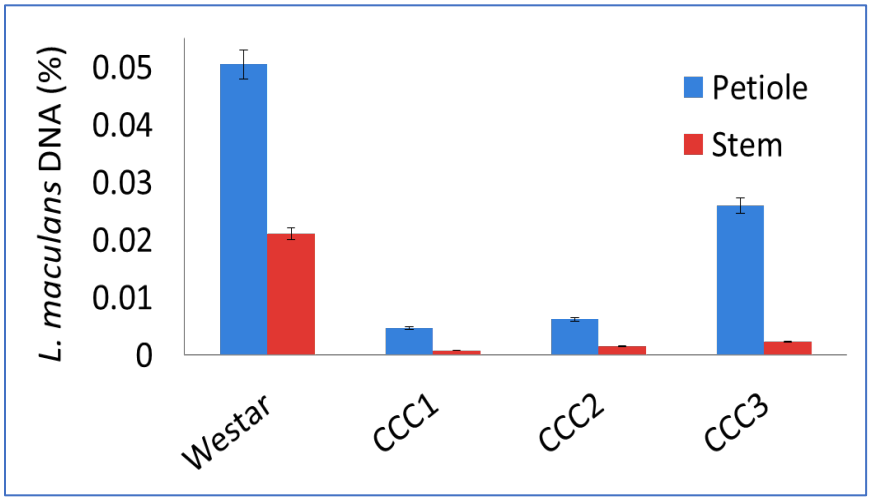


**Fig. 3** The amount of *L. maculans* DNA in the petiole of inoculated Westar and CCCs and in stem tissues adjacent to the petiole at 14 dpi .

**2)** Although blackleg resistance has been widely studied through genetics, molecular mechanisms underlying the host–pathogen interaction remain largely unknown. In this study, transcriptome analysis was carried out



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using a double haploid (DH) *B. napus* line carrying the resistance (R) gene *Rlm1* inoculated with a virulent or avirulent isolate of *L. maculans*. Cotyledon inoculation with either a virulent or avirulent *L. maculans* isolate did not induce systemic acquired resistance (SAR) of the cotyledon on the opposite side challenged with a virulent isolate (**Fig 4**). This indicates that the resistance mediated by *Rlm1* in response to *L. maculans* with *AvrLm1* is only a localized response and cannot be translocated to other parts of the plant. RNA sequencing (RNA-seq) identified a total of 70,709 genes in this DH line by mapping the result to the reference *B. napus* genome, among which 6,999 and 3,015 were differentially expressed genes (DEGs) in the inoculated cotyledon tissue of compatible (susceptible) and incompatible (resistant) interactions. The DH line showed a more pronounced defense response to infection by the virulent isolate, but the proportion of up-regulated DEGs in the resistant interaction (76.1%) was slightly higher than that in compatible interaction (60.7%). Subsequent gene ontology (GO) annotation showed that most of the DEGs were involved in biological processes. Further GO enrichment analysis revealed that a variety of defense-related biological processes, including multiple phytohormone pathways, were commonly enriched among up-regulated DEGs in both susceptible and resistant host reactions, while the significant enrichment of various defense-related GO terms, including the jasmonic acid (JA) and salicylic acid (SA) pathways, were not observed among the down-regulated DEGs in the resistant, but were in susceptible reaction (**Fig 5**), implying that though the defense responses are triggered in both susceptible and resistant host reactions, sufficient levels of JA and SA signals would be required for the activation of resistance to *L. maculans*. On the non-inoculated cotyledon of the same plant, much fewer DEGs were identified, including those involved in the SA pathway implicated in SAR. The comparative study of transcriptome provided a repertoire of candidate genes involved in the regulatory networks for blackleg resistance mediated by the R gene *Rlm1*, and contributed to a better understanding the molecular basis for blackleg resistance by a specific R gene.

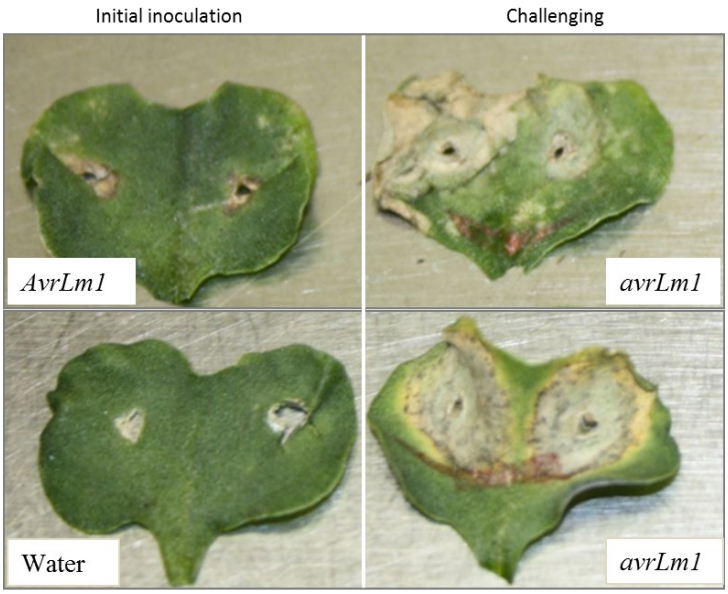


**Fig. 4.** Initial inoculation of cotyledon with an avirulent isolate of *L. maculans* (*AvrLm1*) failed to induce the systemic resistance on the opposite cotyledon challenged with a virulent (*avrLm1*) isolate.

Using the same RNA-seq raw data, the resistance gene in the DH *B. napus* line can be identified in the same region where *Rlm1* from "Quinta" was reported previously (chromosome A07). This range was defined from 13.07 to 22.11 Mb using a BC1 population made from the crosses of F1 plants of DH16516 (susceptible) x DH24288 based on bulked segregant RNA Sequencing (BSR-Seq). *Rlm1* was further fine mapped between 19.6 Mb and 21.7 Mb using a bigger BC1 population consisting of 1247 plants and SNP markers identified using BSR-Seq through Kompetitive Allele-Specific PCR (KASP). BnaA07G28840D, which encodes a cysteine-rich receptor-like protein kinase, was considered as the potential candidate gene for *Rlm1*. The SNP for the gene BnaA07G28840D co-segregated with *Rlm1*. A total of 8 robust SNP markers associated with this *Rlm1* region were identified, which could be useful for efficient introgression of *Rlm1* into canola using marker-assisted selection (MAS) and confirmation of *Rlm1* in canola cultivars for blackleg resistance breeding.

**3)** Although major R genes like *Rlm1* can completely halt blackleg infection at the infection site, the resistance can be overcome rapidly by shifts in the pathogen population. Thus, quantitative resistance (QR) is also of interest against blackleg, especially in western Canada where the crop season is much shorter than many canola-growing regions in the world. The mechanisms underlying QR are unknown. We used RNA-Seq on infected cotyledons of "74-44 BL" to identify genes and gene functions. This is a Canadian cultivar with QR



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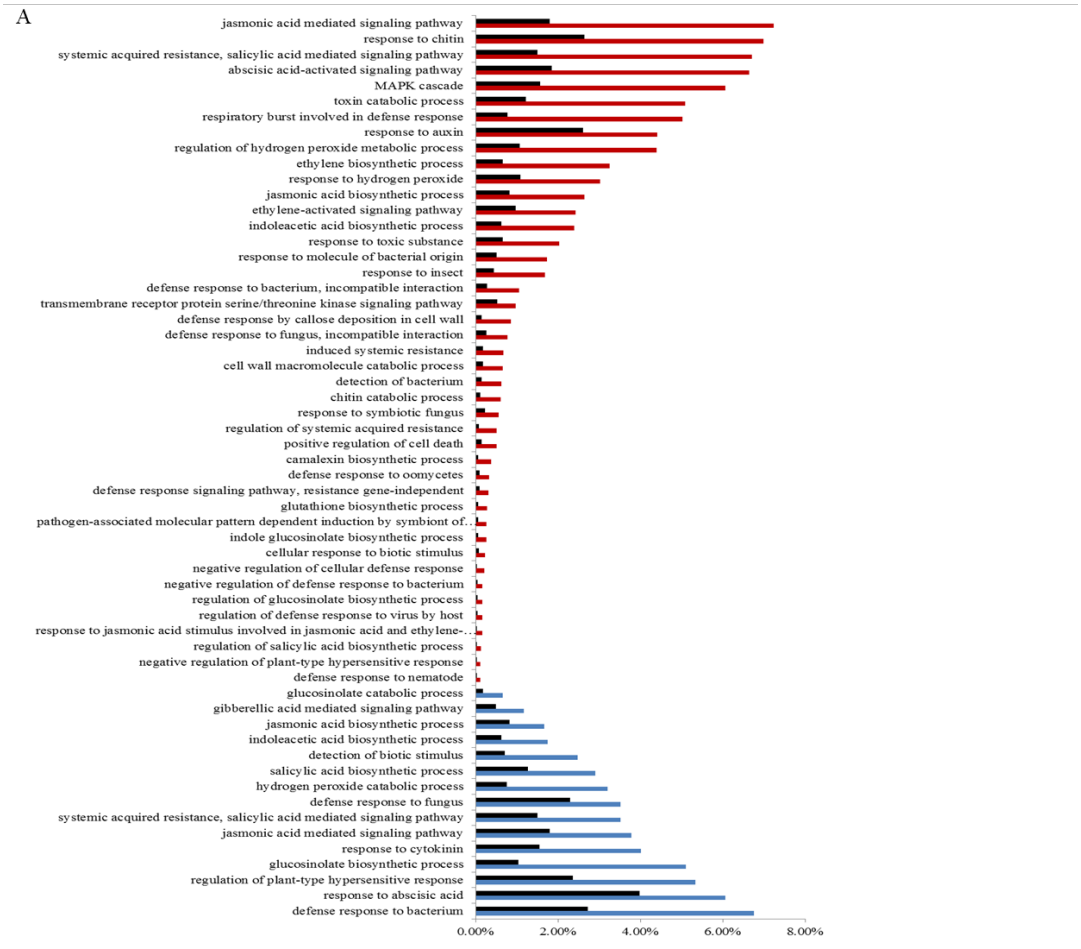
against a range of *L. maculans* isolates without the direct involvement of any major *R* genes. Many genes showed differential expression in inoculated 74-44 BL relative to inoculated Westar, with the highest gene expression for those involved in programmed cell death (PCD), reactive oxygen species (ROS) generation and/or intracellular endomembrane transport (**Fig 6**). Examples include a Bax inhibitor 1 involved potentially in the inhibition of PCD; a development/cell death (DCD) domain containing protein involved potentially in phytohormone-mediated PCD; proteinases and peptidases that may play a role in PCD; a zinc-finger Sec23/ Sec24 and five small GTPases potentially involved in endoplasmic reticulum (ER) to Golgi vesicle traffic and/or signal transduction; five proteins containing WD40 repeats which may mediate protein-protein interactions, PCD and/or vesicle transport within the Golgi apparatus or retrograde Golgi to ER transport and a SecY/Sec61 domain-containing protein putatively involved in incorporation of polypeptides destined for secretion into the ER membrane (**Fig 7**).







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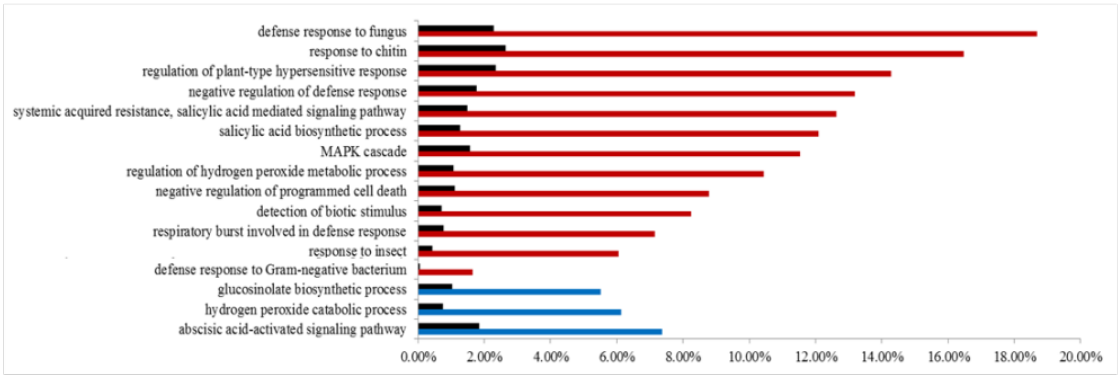


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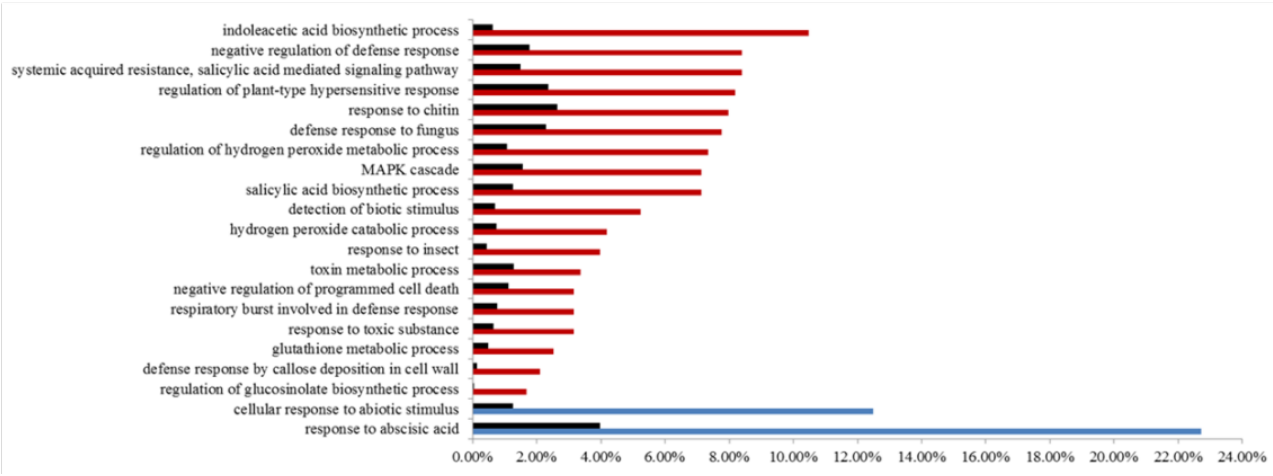


Consistently, Westar displayed symptomless growth of GFP-tagged hyphae over a larger area of inoculated cotyledons than the size of visible lesion. In contrast, hyphae were largely restricted to within the visible lesion in 74-44 BL. In addition, inoculated 74-44 BL cotyledons produced hydrogen peroxide, a trigger of PCD, in a larger area than was colonized by hyphae, while the reverse was true in Westar. These results indicate that QR expressed with 74-44 BL has quite different mechanisms as opposed to those by the major gene *Rlm1*; it appears that the QR is through increased PCD as a way of limiting the biotrophic growth of *L. maculans* in the cotyledons of 74-44BL.

**4)** QR, also known as adult-plant or race nonspecific resistance, has the potential to provide a more durable, if less complete, protection of canola against blackleg. However, the effectiveness of QR may also vary widely in the field, and it has long been suspected that elevated temperatures may negatively affect the expression



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of QR. This is important information since heat waves can happen on the prairies during summer. To test the impact of high temperatures, we assessed the blackleg development on three common canola cultivars (74-44 BL, PV 530 G and 45H29) showing QR, with and without the treatment of 7-h daily exposure to 32°C for one week during early plant flowering under controlled-environment conditions. The impact of elevated temperature on the susceptibility of these cultivars was compared with that under a moderate temperature at 22°C day-time high. Westar was used as a control.

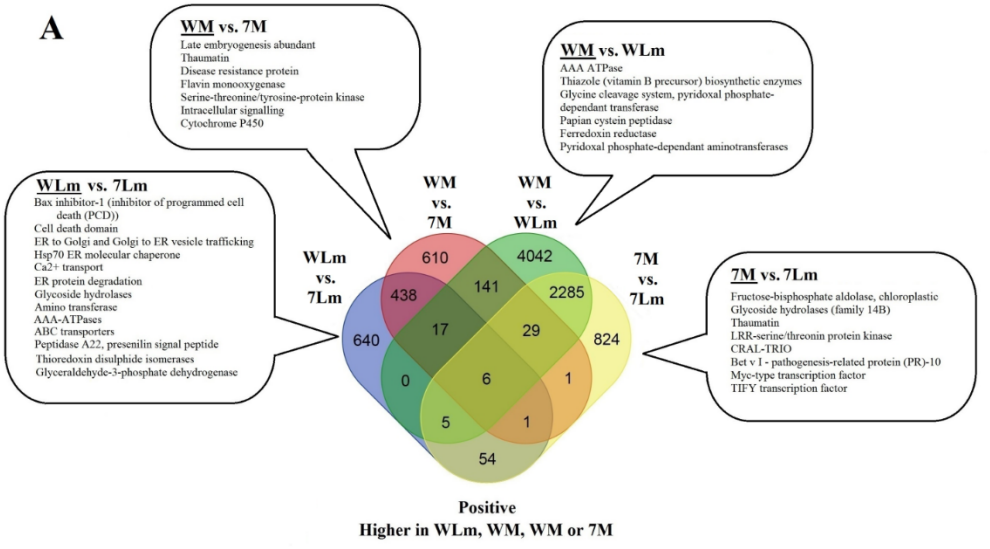





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**Fig 6**. Venn diagrams of upregulated (A) and downregulated (B) DEGs between *L. maculans* inoculated Westar and 74-44, mock inoculated Westar and 74-44, and between mock and inoculated Westar or mock and inoculated 74-44 generated from. DEGs are upregulated in the underlined treatment.

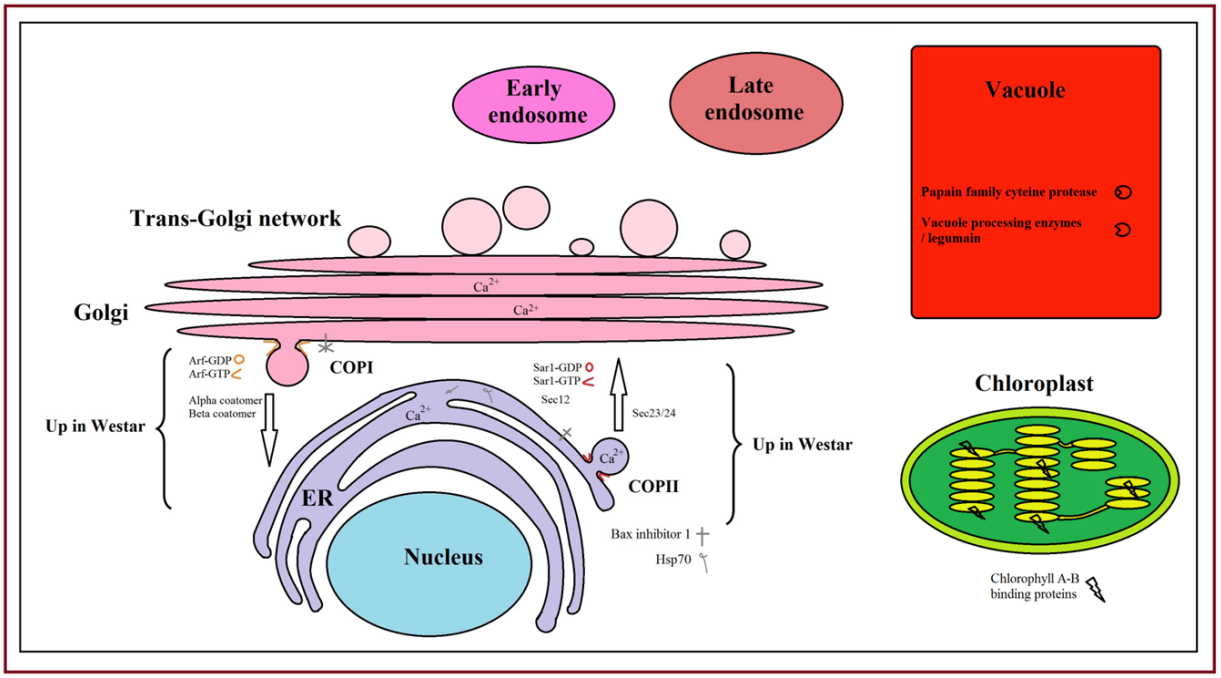


**Figure 7.** Model of how some of the most highly expressed differentially expressed genes (DEGs) may interact. ER: endoplasmic reticulum**.**

When data from both temperatures were pooled, all three QR cultivars showed lower blackleg severity relative to Westar. The elevated temperature often increased blackleg severity on Westar, occasionally on PV 530 G, but generally not on 74-44 BL or 45H29 (**Fig 8, 9, 10, 11**). Our findings suggest that the QR traits are highly useful for blackleg management in western Canada, even with warmer temperatures encountered during rosette to early plant flowering.



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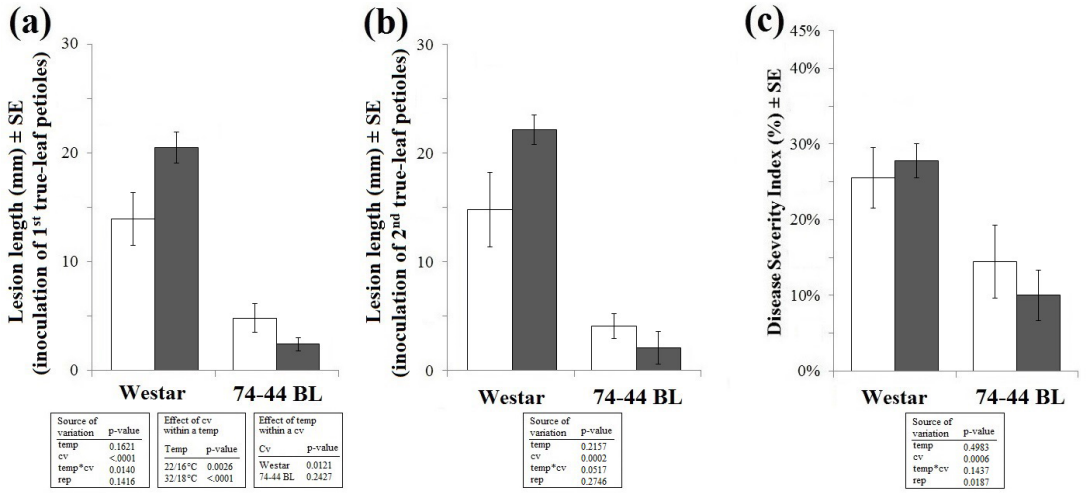


**Fig 8**. Average length of stem lesions originating from the inoculation of first (a) or second (b) true-leaf petioles, at 2 weeks after planting, with *Leptosphaeria maculans*, and disease severity index (c) resulting from the same inoculations in Westar (susceptible control) and 74-44 BL plants subjected to moderate (white bars, 22 °C/16 °C daily high and low) or high (black bars, 32 °C/18 °C daily high and low) temperatures for 1 week at early flowering. The plants had been in the greenhouse for 2 weeks post-inoculation prior to being exposed to these temperature treatments in growth chambers. Mock-inoculated plants were excluded because all such plants had values of zero for all parameters. Statistical highlights are based on a two-way ANOVA with a set at 0.05. cv, cultivar; rep, replication; temp, temperature.

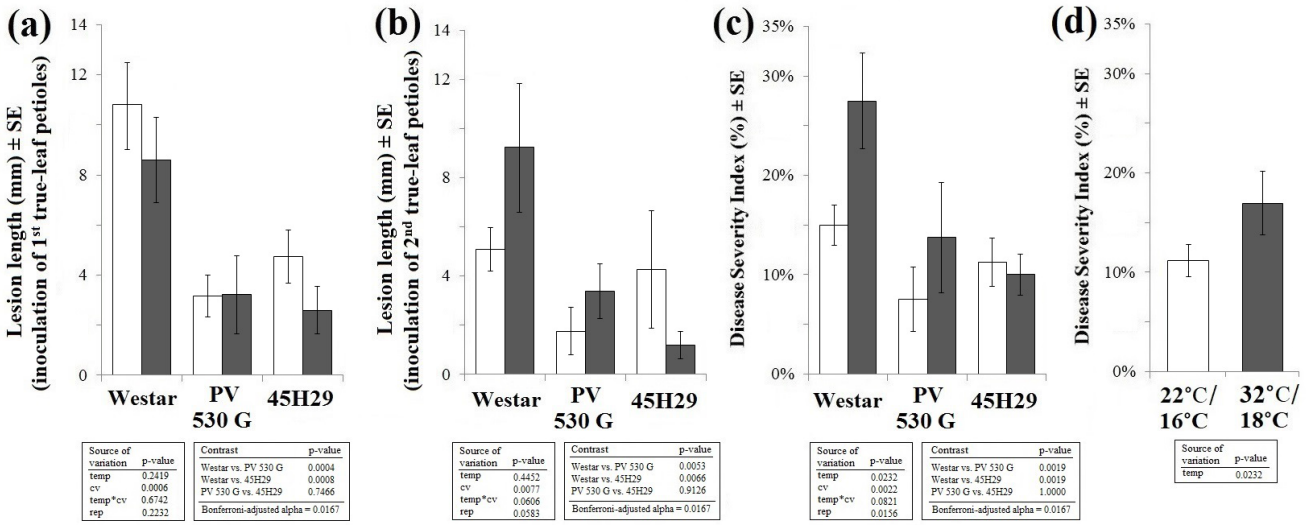





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**Fig 9.** Average length of stem lesions originating from the inoculation of first (a) or second (b) true-leaf petioles, and disease severity index (c) in Westar (susceptible control), PV 530 G and 45H29 plants subjected to moderate (white bars, 22 °C/16 °C daily high and low) or high (black bars, 32 °C/18 °C daily high and low) temperatures. Panel (d) shows the data, pooled for all three cultivars, at moderate and high temperatures. These plants had been in the greenhouse for 2 weeks post-inoculation prior to being exposed to these temperature treatments in growth chambers for 1 week at rosette to early flowering. Mock-inoculated plants were excluded because all such plants had values of zero for all parameters. Statistical highlights are based on a two-way ANOVA, with a set at 0.05. cv, cultivar; rep, replication; temp, temperature.

**Conclusions**

With the extraordinary efforts of all team members, this project was completely successfully. Much of the work was on the leading edge and results were delivered on time, on budget. The most important findings include:

**1)** It was demonstrated that QR is of value in alleviating blackleg impact on canola without the direct involvement of major *R* genes. This is achieved by limiting the spread of fungal hyphae in infected cotyledons further into stems (reduced blackleg incidence) and/or the infection in stem tissues after the pathogen enters it (reduced disease severity). These resistance mechanisms are different from those of sing R genes showed by *Rlm1* that

**2)** induce localized reactions in response to the infection by *L. maculans* carrying *AvrLm1*, which halts the infection immediately by upregulation of many genes involved in the jasmonic acid (JA) and salicylic acid (SA) pathways. This is the first time that molecular mechanism with a specific blackleg resistance gene (*Rlm1*) is identified.

**3)**. Using 74-44 BL, the mechanism underlying QR against blackleg was explored and different genes (as opposed to those involved in *Rlm1*) were found to express differentially, with the highest gene expression associated with those involved in programmed cell death, reactive oxygen species generation and intracellular endomembrane transport. Once confirmed, this will be first report on the molecular mechanisms of QR against plant diseases.

**4)**. The study on the impact of elevated temperature on QR expression indicate that common canola cultivars with a QR background can perform effectively under high-temperature conditions during heat waves and this finding shows that QR traits can be stable under a wide range of field temperatures.



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**Application/impact**

The canola varieties tested for QR are commercial varieties already rated R for blackleg resistance. The information underscores the value of QR by identifying the mechanisms of resistance and understanding the performance under heat-stress conditions. The different modes of action with QR relative to single resistance (*Rlm1*) are unique for understanding the resistance durability.

Overall, the information will be useful for extension messaging in use genetic resistance against blackleg of canola; it is important to develop a strong QR background for blackleg management on the prairies. Putting an effective R gene into a line with good QR will help provide more durable blackleg resistance in western Canada.



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