



**AgrInnovation Program Stream B**

**2017-18 Annual Performance Report**

Name of Recipient: Saskatchewan Canola Development Commission	
Project Title: Canola Disease Management Tools for the Prairies – Blackleg and Sclerotinia	
Project Number: AIP-P032	Period Covered by Report: 2017-04-01 to 2018-03-31
Activity #: 2 Name of Activity: <b>Improving canola resistance against blackleg disease through incorporation of novel resistance genes source from <i>B. napus</i>, <i>B. rapa</i>, and <i>B. oleracea</i></b>	Principal Investigator: CRDA - M. Hossein Borhan, AAFC, Saskatoon Research Centre

**1. Performance Measures.** See Annex A for an explanation of each measure.

Innovation Items	Results Achieved	Provide a description (2-3 paragraphs) for each item produced and describe its importance to the target group or sector. Explain any variance between results achieved and targets. Use plain language.
# of Intellectual property items flowing from the project		
# of new/improved products		
# of new/improved processes or systems		
# of new/improved practices		
# of new varieties		
# of new/improved genetic materials	2	Two new <i>B. napus</i> cv with new R genes against blackleg disease were identified
# of new/ improved gene sequences	1	Position of one new race specific resistance gene was mapped to the lower arm of <i>B. napus</i> chromosome A01
# of improved knowledge	1	More than 1,100 <i>B. napus</i> and <i>B. rapa</i> accessions are characterised for the profile of known Rlm genes (resistance to blackleg). 13 <i>B. rapa</i> accessions that potentially have new R genes were identified.

Information Items	Results Achieved	Provide the complete citation for each item. Please see Annex A for examples.
# of peer reviewed publications		



# of information items	1	Fleury, D, Advancements in finding new sources of resistance genes for blackleg, Canola Digest, Article, 2017/11/20.
# of media reports		
# of information events	1	Borhan, H. Genomics tools and resources for better management of blackleg disease of canola. Blackleg Workshop. Saskatoon, SK. 2018/02/07.
		<b>Provide the # of attendees</b>
# of individuals attending information events	69	
		<b>Provide the # of attendees who intended to adopt new information or technology</b>
# of individuals attending information event who intend to adopt new innovation		
		<b>Provide the name, degree completed and date of completion</b>
# of persons who completed a M.Sc. or Ph.D. during project		

## 2. Executive Summary

The Executive summary contains two parts: Key highlights of activities and scientific results and Success story. Information may be used for internal and external communication purposes. Write for a general audience using plain language. Do not include sensitive or confidential information

**Key Highlights** - This section describes the key activities and final scientific results of an activity/ project in such a way that readers can rapidly become acquainted with a large body of material without having to read it all. Include a brief statement of the problem(s), background information, concise analysis and main conclusions. Suggested length – maximum 1 page.

### Background

Blackleg caused by the fungal pathogen *Leptosphaeria maculans* is a major disease of oilseed rape/canola worldwide. Race-specific resistance genes provide a robust immunity and can readily be incorporated into new varieties allowing for rapid development of resistance cultivars. On the other hand, quantitative resistance, which provides partial immunity, is race non-specific and therefore more durable. Due to their monogenic and often dominant nature, the race-specific resistance genes have been used widely in breeding for resistance to blackleg.

### Problem

Genetic resistance remains the most efficient, practical and cost effective approach for controlling the disease. However, continuous use of a limited number of race-specific resistance genes has contributed to rapid evolution of *L. maculans* and emergence of virulent isolates. The challenge posed by these emerging, virulent isolates of *L. maculans* is likely to be exacerbated by increases in canola acreage and tighter crop rotations, which are driven by the growing demand for canola production. One solution to this challenge is to provide new sources of resistance genes against blackleg disease.



#### Concise analysis and main conclusions

The entire *B. napus* collection at the Plant Gene Resources Canada (PGRC) consist of 466 accessions were screened for resistance to blackleg. Initially three *B. napus* lines named AF229-1 N1, AF272-1 N2 and AF 328-4 N2 were identified as accessions with potential novel R genes against blackleg. Additional genotyping confirmed that lines AF229-1 N1, AF272-1 N2 had the same R gene profile therefore only one of these lines, AF229-1 N1, was proceeded to produce a mapping population. The novel gene from AF328-4-N2 was mapped to the lower arm of chromosome A01 where no other blackleg R gene or blackleg QTL has been reported previously, further supporting the novelty of the resistance. Progenies of AF229-1 N1 population have been phenotyped and genotyping will be conducted to map the location of R gene in AF229-1 N1 line.

From the 551 *B. rapa* lines screened, initially 21 lines showed resistance responses. Up on further screening 10 lines were selected to be selfed and crossed to *B. napus* Topas. Due to incompatibility issues most of the lines produced minimal F1 seed. Five lines 5 with sufficient F1 seed are being selected for further testing.

There are 13 *B. oleracea* accessions listed at the PGRC database. We planted all these lines for pathology test but all of them failed to germinate. Other methods such as first germinating seeds on filter paper also failed.

**Success Story** - A success story presents a significant result or an important milestone achieved. It is intended to showcases achievements in applied research. Focus on research results, successful technology transfer, potential for pre-commercialization, and/or potential impact. A Success Story is not a progress report for each activity (suggested length 2 – 3 paragraphs).

We have conducted an intensive screening of over 1100 accessions of *B. napus* and *B. rapa* to search for novel R genes against blackleg. This has so far lead to the following three outcomes

- 1- Methodology for accurate and rigours screening of Brassica species for blackleg resistance.
- 2- Generating the genetic **profile of known R genes** against blackleg for **over 1100 PGRC accession of Brassica**. This database is invaluable resource for canola breeding programs.
- 3- Two *B. napus* lines with novel R genes against blackleg were identified and preliminary mapping conducted on one of these lines support presence of a **novel broad spectrum R gene against blackleg disease**. Broad spectrum R genes are highly desirable and effective in controlling plant disease due to their monogenic nature.

### **3. Objectives/Outcomes (technical language is acceptable for this section)**

Provide a brief summary that includes introduction, objectives, approach/methodology, deliverables/outputs, results and discussion, and any Ph.D or Master students recruited to work on the project.

#### Introduction

Race specific resistance (R) genes are effective by recognizing target avirulence (Avr) genes in the



pathogens. R-genes have been widely used in breeding for resistance against *Leptosphaeria maculans* because they can be easily deployed into elite breeding lines and protect canola against the disease throughout the plant life cycle. However, continuous use of one or few R-genes creates a selection pressure on the pathogen population which speeds the emergence of virulent isolates. These new virulent isolates carry mutations in the target Avr gene that prevent recognition by the matching plant R-gene. Therefore, new sources of resistance genes are needed to protect the commercial canola cultivars against constantly evolving pathogen populations. Pyramiding several R-genes or rotation of canola cultivars with different sources of resistance will prolong the use of commercial cultivars by reducing selection pressure on the pathogen population.

#### Objectives

Identifying novel genes in *B. napus*, *B. rapa* and *B. oleracea* collection at PGRC

#### Status:

completed

#### Progress in Objectives

In order to maximize the efficiency of screening the *B. napus* and *B. rapa* collections at the PGRC thorough and extensive R gene phenotyping using six Lm isolates was applied to 466 *B. napus* and 551 *B. rapa* accessions. Figure 1 shows the profile of known R genes within the 466 *B. napus* accessions. Lines with potential novel R gene were selfed for homozygosity and tested with up to additional 40 isolates. This further vigorous screening confirmed 3 *B. napus* lines named AF229-1 N1, AF272-1 N2 and AF 328-4 N2 with novel R genes. Two of these lines, AF229-1 N1, AF272-1 N2, had the same genotype of R gene and were considered as being the same genetic material with regard to resistance against blackleg. To map the location of new blackleg R genes in these lines F2 and BC1 populations were made by crossing , AF229-1 N1 and AF328-4 N2 to the susceptible *B. napus* cv Topas respectively. The broad-spectrum R gene in AF328-4 N2, was mapped to the lower arm of A01 chromosome of the *B. napus* genome distant from the recently reported positions of major blackleg QTL loci (Larkan et al., 2106; Raman et al., 2016), further supporting the novelty of the resistance. Progenies of the AF229-1 N1 x Topas population have been phenotyped and the position of R gene will be mapped using Brassica SNP arrays.

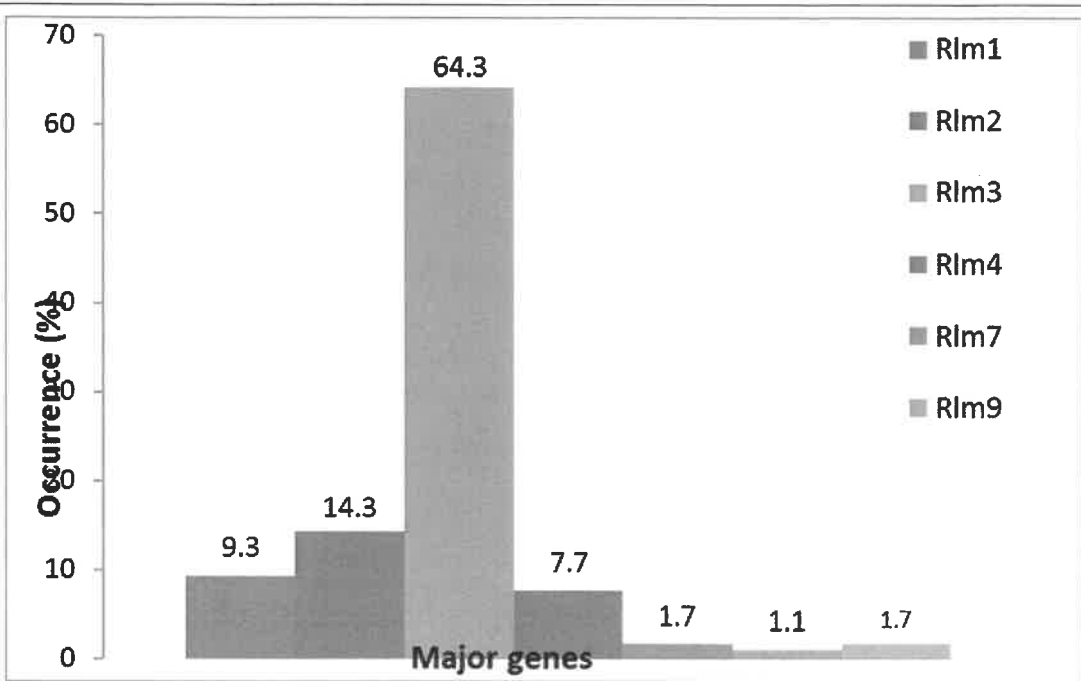


Fig1. Profile of known blackleg resistant genes present in Brassica napus L. from the Plant Genetic Resources Center, Canada.

Of the 551 *B. rapa* lines, 21 lines with known R gene and potential presence of new R genes were carried forward. 10 of these lines have been used in crosses with *B. napus*. Seed production has been hindered by self incompatibility issues.

All lines 13 *B. oleracea* accessions currently available from the PGRC were planted in soil and failed to germinate. Attempt to germinate seeds on paper filter also failed.

#### 4. Issues

- Describe any challenges or concerns faced during the project. How were they overcome or how do you plan to overcome?
- Describe any potential changes to the work plan and the budget. How were or how will they be managed?

*B. oleracea* seeds from PGRC has lost their vigour and did not germinate using different approaches. Also self incompatibility of *B. rapa* lines is a limiting factor

#### 5. Lessons Learned:

Describe the key lessons learned gained as a result of executing the project (e.g., a more efficient approach to performing a specific task for activity / project).



We have explored different approaches to encourage germination of *B. oleracea* lines and all have failed.

#### 6. Future Related Opportunities:

Describe the next steps for the innovation items produced by the activity/project. Is additional research required? Is there potential for commercialization or adoption?

To date presence of a **novel R gene** in one of the two *B. napus* accessions (**AF328-4 N2**) originally identified as lines with potential novel R gene has been further supported by mapping. This gene, located on the chromosome A1, behaves as a **broad spectrum** R gene as it shows resistance response to majority of isolates being tested and as such will be highly valuable in **controlling blackleg disease in western Canada**.



AgrInnovation Program Stream B

## 2017-18 Annual Performance Report

Name of Recipient: Saskatchewan Canola Development Commission	
Project Title: Canola Disease Management Tools for the Prairies – Blackleg and Sclerotinia	
Project Number: AIP-P032	Period Covered by Report: 2017-04-01 to 2018-03-31
Activity #:3 Name of Activity: Identifying novel resistance genes from canola relatives and developing canola germplasm with multiple resistance genes sourced from <i>B. nigra</i> , <i>B. juncea</i> , and <i>B. carinata</i>	Principal Investigator: Genyi Li and Dilantha Fernando, University of Manitoba

**1. Performance Measures.** See Annex A for an explanation of each measure.

Innovation Items	Results Achieved	Provide a description (2-3 paragraphs) for each item produced and describe its importance to the target group or sector. Explain any variance between results achieved and targets. Use plain language.
# of Intellectual property items flowing from the project	0	
# of new/improved products	0	
# of new/improved processes or systems	N/A	
# of new/improved practices	N/A	
# of new varieties	N/A	
# of new/improved genetic materials	8	Materials containing novel resistance genes have been developed from hexaploidy species (AABBCC) and <i>B. juncea</i> (AABB). Currently eight lines have been selected from indoor testing.
# of new/ improved gene sequences	4	Blackleg resistance genes on chromosome N7 have been sequenced and their function has been confirmed.  3 genes were identified for pathogenicity: LmPf2, Lm60S and LmMbpA. 1) LmMbpA, an APSES transcription factor that is required for development and is also important for complete pathogenicity in <i>L. maculans</i> against <i>B. napus</i> . A mutation in this gene in <i>L. maculans</i> showed that it is not an essential factor for pathogenicity but required for optimal levels, as



Innovation Items	Results Achieved	Provide a description (2-3 paragraphs) for each item produced and describe its importance to the target group or sector. Explain any variance between results achieved and targets. Use plain language.
		<p>mutants were slightly reduced in pathogenicity when inoculated on B. napus Westar.</p> <p>2) Lm60S, an acidic ribosomal P2 protein, was shown to be required for disease development in B. napus Westar. The disease symptoms were significantly reduced when the host was inoculated with the mutant compared to that inoculated with the wild type JN3. Interestingly, there was still a bit of necrosis and lesion formation, but there was no formation of pycnidia at the site of inoculation.</p> <p>3) LmPf2, a transcriptional regulator from the C6-Zn finger cluster family, was shown to be not only required for L. maculans disease establishment in B. napus, but was illustrated to be required for optimal expression of effector proteins. The host defense response to the L. maculans mutant indicated that there was no longer a defense response elicited when in contact with the mutant pathogen. This particular protein has been reported in other fungal isolates for being extremely important for necrotrophic development and it appears that this hold true in L. maculans.</p>
# of improved knowledge	2	<p>We have learned through our research that LmPf2 is a transcription factor that is essential for L. maculans JN3 disease development in B. napus. This is important information because this particular gene could be potentially used as an ideal target for generating synthetic fungicides against B. napus. In addition in host RNAi silencing could also be used to generate host plants that target this particular L. maculans fungal gene. Since RNAi can be carried through to progeny it is possible to generate B. napus lines that target LmPf2 potentially eliminating disease that could be caused by many of the L. maculans races present in the field.</p> <p>Increased understanding of how L. maculans causes disease - The most interesting finding was through using the ΔLmPf2 (T-DNA mutant). This mutant exhibited no disease when inoculated on Westar, or HR response when inoculated onto resistant cultivars. When we examined the defense gene expression, the SA, JA and ET responsive genes along with RBOHD, were significantly reduced when Westar was inoculated with these mutants compared to inoculation with wt. In addition, we found that LmPf2 transcription factor was required for the transcription of StuA, an APSES transcription</p>





Innovation Items	Results Achieved	Provide a description (2-3 paragraphs) for each item produced and describe its importance to the target group or sector. Explain any variance between results achieved and targets. Use plain language.
		factor that has been shown to regulate effector production. When we examined effector production there was also a significant reduction in expression of AvrLm1, 4-7 and AvrLm6. Our findings indicate that LmPf2 is another measure of control to ensure optimal expression of specific effectors at the right time during infection.

Information Items	Results Achieved	Provide the complete citation for each item. Please see Annex A for examples.
# of peer reviewed publications		
# of information items		
# of media reports		
# of information events		
		<b>Provide the # of attendees</b>
# of individuals attending information events		
		<b>Provide the # of attendees who intended to adopt new information or technology</b>
# of individuals attending information event who intend to adopt new innovation		
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# of persons who completed a M.Sc. or Ph.D. during project		

## 2. Executive Summary

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concise analysis and main conclusions. Suggested length – maximum 1 page.

Blackleg resistance is exploited and used to reduce yield losses in canola production. To develop new cultivars with high levels of resistance to blackleg, collecting information about the R gene sequences and their function is very important. The blackleg resistance genes on chromosome N7 have been studied for three decades and none of those genes on N7 has been sequenced due to the structure complexity of this chromosome. Recently, we sequenced and functionally confirmed the R gene sequences on N7. This is a significant progress in canola blackleg research.

Novel blackleg resistance has been introduced into canola from hexaploidy species and mustard (*Brassica juncea*). More than 10 advanced backcrossing lines (BC5 and BC6) have been produced in a susceptible cultivar Westar background and all these lines showed excellent resistance to aggressive isolates of blackleg pathogen. These novel genes are being introduced into breeding materials at the University of Manitoba and will be used in a few years. These materials with novel blackleg resistance genes will be available to Canadian seed companies.

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### 3. Objectives/Outcomes (technical language is acceptable for this section)

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In Dr. Li's lab, there are two Ph.D. students and two research associates who are working on blackleg resistance gene identification and transferring novel resistance genes from canola relatives into canola in this reported period.

Novel blackleg resistance genes from hexaploid species (AABBCC) and *B. juncea* (AABB) were introduced into the susceptible *B. napus* (AACC) cultivar 'Westar'. We obtained BC6 and BC5F2 plants which were the same as 'Westar' with regard to their morphology and some introgression lines showed good seed set. After testing these advanced generation, we found that the novel resistance gene introduced from hexaploids into *B. napus* was highly resistant to highly virulent blackleg fungal isolates. Eight lines have been selected and will be tested in blackleg nursery in 2018.

We produced BC5 and BC5F2 plants in segregation of blackleg resistance. The seed set in most



introgression lines were good. Eight resistant lines have been selected for field testing in 2018.

Using the gene cloning information in canola, we develop molecular markers to test the gene introgression lines developed from the hexaploid species. Interestingly, we found that in some introgression lines, molecular markers showed linkage with the novel blackleg resistance genes. The results suggested that we might develop closely linked molecular markers for the novel blackleg resistance genes that can be used in molecular marker assisted selection of the novel resistance genes in canola breeding. This claim needs to be confirmed when we will test more introgression lines and the following backcrossing and selfing progeny.

Using the gene sequences we obtained, molecular markers have been developed for these novel R genes introduced from hexaploid and *B. juncea*.

In Dr. Fernando's lab we focused on identifying the role and key functions of pathogenicity factors in disease and host interactions to provide new insights into managing the disease. Therefore, the objective of this project is to elucidate the role and function of pathogenicity genes involved in disease by generating gene disruption mutants utilizing *Agrobacterium tumefaciens*-mediated random insertional mutagenesis (ATMT).

ATMT has been utilized for generating mutants in many different fungi including *L. maculans*. In this study, the *A. tumefaciens* strain Agl1 harboring the pKHT vector was utilized to generate random mutations in the JN3 (AvrIm1, 2, 4, 5, 6, 7, and AvrIm8) *L. maculans* isolate. The pKHT carries the T-DNA portion that inserts randomly into the genome, where selection of the fungal isolates with TDNA inserts is through the hygromycin antibiotic selection marker. The transformation was carried out by mixing 4.5 ml of induced *A. tumefaciens* Agl-1 with 2.25ml of spores. The mixture was then centrifuged and supernatant was removed and 950  $\mu$ l of induction medium (IM) was used to resuspend the pellet. The concentrated mixture was then spread onto IM plates and incubated in the dark for 3 days. After incubation, the plates were overlaid with molten 10% V8 agar (hygromycin 100  $\mu$ g/ml, cefotaxime 200  $\mu$ g/ml). Plates were then placed in the light at room temperature for 10-21 days. Fungal isolates that grew were plated 2x on 10% V8 (hygromycin 100  $\mu$ g/ml, cefotaxime 200  $\mu$ g/ml) to ensure mutant stability. In a total of 3 transformations, 120 transformants were generated, all of which carried the hygromycin cassette as detected by PCR. 80 of these transformants exhibited single insertions as indicated by Southern hybridization. Of these 80 transformants, only four transformants exhibited a reduction in pathogenicity towards *Brassica napus* (Westar). To determine where the T-DNA was located in these four mutants, thermo-interlaced asymmetric (TAIL)-PCR was used to obtain a DNA amplicon comprised of the T-DNA border with flanking genomic region. The PCR amplicon was then sequenced and BLAST was utilized to determine the genomic location of the T-DNA. The BLAST results of the sequences obtained indicated that only three of the four mutants had T-DNA inserts corresponding to an *L. maculans* gene. The insertion site for each of the three mutants were as follows: One mutant, termed  $\Delta$ Lm60S had the T-DNA inserted in the 60S acidic ribosomal protein, the second mutant, termed  $\Delta$ LmHp, had an insertion located in a gene encoding a hypothetical protein and the third mutant termed  $\Delta$ LmMbpA, illustrated an insertion in the gene encoding the APSES transcription factor MbpA.

We focused our attention on the  $\Delta$ LmHp mutant, as this isolate was quite interesting as it exhibited some shared phenotypic qualities (no disease on host, little fungal biomass)



accumulation in planta) that were reported previously in an LmStuA silenced (RNAi) *L. maculans* isolate. When we looked closer at the protein sequence, we found that it shared 79% identity with another C6-Zn finger cluster protein termed AbPf2 in *Alternaria brassicicola*. These types of proteins are unique to fungi, and are DNA binding proteins that have been shown to be required for transcriptional regulation of fungal genes. AbPf2 was shown to be important for necrosis and required for effector production. In addition plant defense expression of genes associated with host responses to chitin, jasmonic acid, ethylene, wounding, and oxidative stress were expressed at lower levels in the Brassica host when inoculated with the AbPf2 mutant compared to being inoculated with the wild-type isolate. We then hypothesized that this hypothetical protein may be required for pathogenicity because and somehow required for StuA expression and may also be required for effector gene transcription. When we examined in vivo expression of fungal genes there was a significant reduction of LmStuA expression, along with the expression of AvrLm1, AvrLm4-7 and AvrLm6 at 7 and 11 dpi in the LmPf2 mutant compared to JN3, indicating a connection between LmPf2 and LmStuA and effector production. We also detected a significant reduction in rbohD expression (ROS-oxidative burst) and a reduced expression in SA, JA and ET associated genes. It appears as though *B. napus* (Westar) no longer detects this isolate or elicits any type of defense response, in fact, when we tested the isolates on resistant cultivars, the response was the same as the susceptible Westar, where no HR response was visible. Taken together, it appears that LmPf2 may be required for necrotrophic development in planta, a stage in which both StuA and effector expression clearly rely on.

#### 4. Issues

- Describe any challenges or concerns faced during the project. How were they overcome or how do you plan to overcome?
- Describe any potential changes to the work plan and the budget. How were or how will they be managed?

N/A.

#### 5. Lessons Learned:

Describe the key lessons learned gained as a result of executing the project (e.g., a more efficient approach to performing a specific task for activity / project).

N/A.

#### 6. Future Related Opportunities:



Describe the next steps for the innovation items produced by the activity/project. Is additional research required? Is there potential for commercialization or adoption?

Plant materials with novel blackleg resistance genes will be made available to canola industry through signing MTA with the University of Manitoba.



**AgrInnovation Program Stream B**

**2017-18 Annual Performance Report**

Name of Recipient: Saskatchewan Canola Development Commission	
Project Title: Canola Disease Management Tools for the Prairies – Blackleg and Sclerotinia	
Project Number: AIP-P032	Period Covered by Report: 2017-04-01 to 2018-03-31
Activity #: 4 Name of Activity: <b>Genome-wide association mapping of quantitative resistance against blackleg disease</b>	Principal Investigator: M. Hossein Borhan, AAFC, Saskatoon Research Centre

**1. Performance Measures.** See Annex A for an explanation of each measure.

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# of Intellectual property items flowing from the project		
# of new/improved products		
# of new/improved processes or systems		
# of new/improved practices		
# of new varieties		
# of new/improved genetic materials	2	58 B. napus lines with quantitative resistance to blackleg disease were identified and presence of APR was confirmed.  A population of 120 comprising of B. napus accessions with QTL and susceptible lines have been developed. Final round of pathology test confirmed the presence of quantitative resistance loci against blackleg in these population.
# of new/ improved gene sequences		
# of improved knowledge		

<b>Information Items</b>	<b>Results Achieved</b>	<b>Provide the complete citation for each item. Please see Annex A for examples.</b>
# of peer reviewed publications		



# of information items		
# of media reports		
# of information events		
		<b>Provide the # of attendees</b>
# of individuals attending information events		
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<p>Two types of resistance have been described against blackleg. One that is effective at preventing disease at the cotyledon stage (major resistance genes) and the other is resistance of adult plants to latent systemic infection (quantitative resistance). Cotyledon resistance is race-specific and is often the result of a single resistance gene in the plant that is able to recognize a matching avirulence gene present in the pathogen. Another type of resistance is adult plant resistance that is quantitative (polygenic) and race non-specific. Quantitative resistance, while difficult to incorporate into canola lines, it is more durable against the evolving pathogen races. Through this project a collection 58 spring-type B. napus with adult plant resistance (APR) against blackleg disease was identified. These lines offer a source of quantitative resistance against blackleg disease.</p>
<p><b>Success Story</b> - A success story presents a significant result or an important milestone achieved. It is intended to showcase achievements in applied research. Focus on research results, successful technology transfer, potential for pre-commercialization, and/or potential impact. A Success Story is not a progress report for each activity (suggested length 2 – 3 paragraphs).</p>
<p>Adult plant resistance (APR) against blackleg disease of canola is a durable form of resistance and</p>



protects canola against the *L. maculans* infection. The spring type *B. napus* accessions with APR, identified during the course of this project facilitate rapid incorporation of the highly desirable APR into commercial canola cultivars.

### 3. Objectives/Outcomes (technical language is acceptable for this section)

Provide a brief summary that includes introduction, objectives, approach/methodology, deliverables/outputs, results and discussion, and any Ph.D or Master students recruited to work on the project.

**Introduction:** Two types of resistances have been described against blackleg. One that is effective at preventing disease at the cotyledon stage (major resistance genes) and the other is resistance of adult plants to latent systemic infection (quantitative resistance). Cotyledon resistance is race-specific and is often the result of a single resistance gene in the plant that is able to recognize a matching avirulence gene present in the pathogen. Another type of resistance is adult plant resistance that is quantitative (polygenic) and race non-specific, hence is more durable against the evolving pathogen races (2).

**Objective:** to tightly associate SNP markers to loci controlling adult plant resistance and define the underlying genetic architecture of this durable resistance to blackleg in *B. napus*.

Status: completed

**Methodology:** To eliminate variation caused by both the environment and the mixed, undefined population of *L. maculans* field isolates, we developed and applied a growth chamber (GC) based assay for quantitative resistance to blackleg (BL-QTL). This method relies on inoculation with highly virulent *L. maculans* isolates and the evaluation of adult plant response in cultivars that are fully susceptible at the cotyledon stage (they lack race specific resistance against the *L. maculans* isolate used for inoculation). Disease ratings was performed on adult plants using a scale of 0 to 10 based on the discoloration of a cross section at the crown (0: no lesion; and 10: dead plant).

**Results:** In order to confirm the phenotype of GWA population, prior to proceeding with mapping the quantitative resistance loci, we formed a population consisting of 58 lines with quantitative resistance and an equal number of susceptible lines from the spring type *B. napus* collection. The entire GWA mapping population was phenotyped to confirm the QTL response.

Reference:

Larkan, N. J., et al. (2016). Multi-environment QTL studies suggest a role for cysteine-rich protein kinase genes in quantitative resistance to blackleg disease in *Brassica napus*. *BMC Plant Biology*, 16(1), 183. <http://doi.org/10.1186/s12870-016-0877-2>

### 4. Issues

- Describe any challenges or concerns faced during the project. How were they overcome or





how do you plan to overcome?

- Describe any potential changes to the work plan and the budget. How were or how will they be managed?

NA

**5. Lessons Learned:**

Describe the key lessons learned gained as a result of executing the project (e.g., a more efficient approach to performing a specific task for activity / project).

NA

**6. Future Related Opportunities:**

Describe the next steps for the innovation items produced by the activity/project. Is additional research required? Is there potential for commercialization or adoption?



**AgrInnovation Program Stream B**

**2017-18 Annual Performance Report**

Name of Recipient: Saskatchewan Canola Development Commission	
Project Title: Canola Disease Management Tools for the Prairies – Blackleg and Sclerotinia	
Project Number: AIP-P032	Period Covered by Report: 2017-04-01 to 2018-03-31
Activity #: 5 Name of Activity: Transcriptomic analysis of the <i>Leptosphaeria maculans</i> - (blackleg-canola) interaction to identify resistance genes in canola and avirulence factors in <i>L. maculans</i>	Principal Investigator: Richard Bélanger, University of Laval.

**1. Performance Measures.** See Annex A for an explanation of each measure.

Innovation Items	Results Achieved	Provide a description (2-3 paragraphs) for each item produced and describe its importance to the target group or sector. Explain any variance between results achieved and targets. Use plain language.
# of Intellectual property items flowing from the project		
# of new/improved products		
# of new/improved processes or systems		
# of new/improved practices		
# of new varieties		
# of new/improved genetic materials		
# of new/ improved gene sequences	1	Complete sequences and profiles of aquaporins in canola and five closely related Brassicaceae species
# of improved knowledge	4	<ol style="list-style-type: none"> <li>1. An analytical pipeline was designed through which classically secreted proteins along with their key conserved motifs were identified in 12 common plant pathogens including <i>L. maculans</i> (11 fungi and one oomycete)</li> <li>2. Differentially expressed genes were identified in <i>L. maculans</i> during biotrophy and necrotrophy phases</li> <li>3. Analysis of aquaporins performed in this project will be helpful for enhancing our understanding</li> </ol>



Innovation Items	Results Achieved	Provide a description (2-3 paragraphs) for each item produced and describe its importance to the target group or sector. Explain any variance between results achieved and targets. Use plain language.
		<p>and related physiological processes that could be exploited in breeding programs of stress-tolerant cultivars</p> <p>4. Transcriptome data analysis will provide a basis to understand molecular mechanisms involved in blackleg disease development in canola.</p>

Information Items	Results Achieved	Provide the complete citation for each item. Please see Annex A for examples.
# of peer reviewed publications	1	Sonah, H., Deshmukh, R., Labbe, C. & Belanger, R.R. 2017. Analysis of aquaporins in Brassicaceae species reveals high level of conservation and dynamic role against biotic and abiotic stress in canola. Nature Scientific Reports 2017 Jun 5;7(1):2771. doi: 10.1038/s41598-017-02877-9
# of information items		
# of media reports		
# of information events	5	<ol style="list-style-type: none"> <li>1. Fernando D. Understanding Blackleg Resistance. CanolaPALOOZA. Lacombe, Alberta. 2017/06/27</li> <li>2. Fernando, D. Advancements made in blackleg research in oilseed rape in the University of Manitoba. Seminar. University of Hertfordshire, United Kingdom. 2017/05/09</li> <li>3. Fernando, D. Diseases, host and pathogen interactions. Seminar. University of Ruhuna, Sri Lanka. 2017/05/17</li> <li>4. Fernando, D. Canola Industry Blackleg Labeling Model: a process for successful blackleg mitigation and reducing R-gene breakdown. Western Forum on Pest and Disease Management. Winnipeg, MB. 2017/10/26</li> </ol>
		<b>Provide the # of attendees</b>
# of individuals attending information events	475	<ol style="list-style-type: none"> <li>1. 300</li> <li>2. 50</li> <li>3. 50</li> <li>4. 75</li> </ol>



		<b>Provide the # of attendees who intended to adopt new information or technology</b>
# of individuals attending information event who intend to adopt new innovation	n/a	
		<b>Provide the name, degree completed and date of completion</b>
# of persons who completed a M.Sc. or Ph.D. during project	1	Xuehua Zhang, PhD, October 2016

## 2. Executive Summary

The Executive summary contains two parts: Key highlights of activities and scientific results and Success story. Information may be used for internal and external communication purposes. Write for a general audience using plain language. Do not include sensitive or confidential information

**Key Highlights** - This section describes the key activities and final scientific results of an activity/project in such a way that readers can rapidly become acquainted with a large body of material without having to read it all. Include a brief statement of the problem(s), background information, concise analysis and main conclusions. Suggested length – maximum 1 page.

### 1. Computational Prediction of Effector Proteins in Fungi: Opportunities and Challenges

Effector proteins are mostly secretory proteins that stimulate plant infection by manipulating the host response. Identifying fungal effector proteins and understanding their function is of great importance in efforts to curb losses to plant diseases. In this work, we developed a unique pipeline where an exhaustive list of classically secreted proteins along with their key conserved motifs found in 12 common plant pathogens including *L. maculans* were provided. Combining available pipelines with the ever increasing structural, genomic and transcriptomic data will lead to a better prioritization strategy where the most promising effectors can be rapidly targeted for future analyses aimed at a better understanding of pathogenesis processes in canola-*L. maculans* interaction

### 2. Gene expression dynamics of blackleg disease development revealed by Dual-RNAseq approach in differential lines of canola (*Brassica napus*)

In that study, transcriptome profiling of *L. maculans* was performed in an effort to understand and define the pathogenicity genes that govern both the biotrophic and the necrotrophic phase of the fungus, as well as those that separate a compatible from an incompatible interaction. Analysis of 1.6 billion Illumina reads readily identified differentially expressed genes that were over represented by candidate secretory effector proteins, CAZymes, and transcription factors (TFs). Comparisons between the compatible and incompatible interactions led to the identification of 28 effector proteins whose chronology and level of expression suggested a role in the establishment and maintenance of biotrophy with the plant. These included all known *Avr* genes of isolate D5 along with nine newly characterized effectors. In conclusion, comparison of the transcriptome of *L. maculans* during compatible and incompatible interactions has led to the identification of key pathogenicity genes that regulate not only the fate of the interaction but also lifestyle transitions of the fungus.



### 3. Analysis of aquaporins in Brassicaceae species reveals high level of conservation and dynamic role against biotic and abiotic stress in canola

In this study, genome-wide identification, distribution, and characterization of AQPs were determined in *Arabidopsis lyrata*, *Capsella grandiflora*, *C. rubella*, *Eutrema salsugineum*, *Brassica rapa*, *B. oleracea*, and *B. napus* (canola). Characterization of distinctive AQP features showed a high level of conservation in spacing between NPA-domains, and selectivity filters. Interestingly, TIP3s were found to be highly expressed in developing seeds, suggesting their role in seed desiccation. Analysis of available RNA-seq data obtained under biotic and abiotic stresses led to the identification of AQPs involved in stress tolerance mechanisms in canola. In addition, analysis of the effect of ploidy level, and resulting gene dose effect performed with the different combinations of Brassica A and C genomes revealed that more than 70% of AQPs expression were dose-independent, thereby supporting their role in stress alleviation. This first in-depth characterization of Brassicaceae AQPs highlights transport mechanisms and related physiological processes that could be exploited in breeding programs of stress-tolerant cultivars

**Success Story** - A success story presents a significant result or an important milestone achieved. It is intended to showcase achievements in applied research. Focus on research results, successful technology transfer, potential for pre-commercialization, and/or potential impact. A Success Story is not a progress report for each activity (suggested length 2 – 3 paragraphs).

While we are certainly not at the stage of technology transfer, we feel that our fortuitous discovery of the role of AQPs in canola has opened the door to some potential exciting achievements. Case in point, our hypothesis to exploit specific AQPs to alleviate stress in canola has led to a strategic grant totalling over 500,000\$ in research support (see Future Research Opportunities).

### 3. Objectives/Outcomes (technical language is acceptable for this section)

Provide a brief summary that includes introduction, objectives, approach/methodology, deliverables/outputs, results and discussion, and any Ph.D or Master students recruited to work on the project.

Blackleg disease (stem canker) caused by *Leptosphaeria maculans* is one of the major constraints to canola (*Brassica napus*) production worldwide. Management of blackleg disease includes crop rotations, seed treatment and fungicide applications, and, preferably, disease-resistant cultivars, arguably the most effective approach. The infection process is highly dependent on host recognition and molecular cross talk between the host and the pathogen where pathogenicity-related genes play an important role. However, any given host-pathogen interaction is a very complex phenomenon, which makes it difficult to understand the factors dictating compatibility or incompatibility. Expression profiling during the development of disease is an



effective approach to better understand the pathogenesis process. Current improvements in sequencing technologies have provided new opportunities to evaluate gene expression under different conditions by sequencing the entire transcriptome. In our study, we analyzed the RNA-seq transcriptome profiling of *L. maculans* inoculated on susceptible and resistant canola lines at five developmental stages with five biological replications. A particular emphasis was placed on the identification of DEGs including effectors, TFs, and CAZymes, and molecular pathways and metabolic processes involved in the pathogenesis process during blackleg disease development in canola. In addition, we studied the complete transcriptome of the same canola lines and, notwithstanding the usual defense responses, we stumbled upon interesting observations regarding aquaporin expression and their role in stress that we exploited further in this project.

The first objective of this study was to identify effectors using different tools and pipelines.

The second objective was to compare *L. maculans* responses against compatible and incompatible host genotypes in order to obtain a more precise definition of the virulence factors expressed by *L. maculans* over time.

A third objective was to compare canola responses to identify genes involved in resistance/susceptibility and this led to an innovative approach where we investigated the role of aquaporins (AQPs), and their expression in stress (e.g. *L. maculans*) alleviation in canola.

Whole genome sequencing of *L. maculans* have provided valuable resources. Predicted protein sequences from the *L. maculans* genome were retrieved from the JGI MycoCosm (<http://genome.jgi.doe.gov/Lepmu1/Lepmu1.home.html>). SignalP and TMHMM software tools along with Secretool pipeline were used to predict small-secretory proteins. To prioritize candidate effector genes, the entire secretome was analyzed by EffectorP a machine learning method optimized for the fungal effector prediction. Similarly, identification, distribution and characterization of effectors was performed in 11 highly devastating plant pathogenic fungi.

A total of 552 SSPs were identified in *L. maculans* using SignalP and TMHMM software tools along with Secretool pipeline. Following further analyses, 134 SSPs were prioritized as high confidence CSEPs based on the results obtained with EffectorP software. Similarly expected numbers of CSEPs were observed in the exhaustive list of SSPs from 11 other fungi.

To study the transcriptomic responses of *L. maculans*, D5 strain was inoculated on compatible host Topas-wild and incompatible host Topas-Rlm2. Leaf samples at five stages were harvested and used for RNA extraction and subsequent RNA-seq library preparation. RNA-seq reads obtained with HiSeq2500 were quality-checked with fastqc, and read processing was performed by using Trimmomatic software. Processed reads were aligned to the *L. maculans* genome and transcriptome with Tophat. The gene expression level was estimated as the number of Fragments (reads) per kilobase of transcript per million mapped reads (FPKM) using only uniquely mapped reads. The differentially expressed genes (DEGs) were identified using four different tools including Cuffdiff, EdgeR, Deseq2 and CLC workbench. We used FDR <0.0001 and the absolute value of  $\log_2(\text{FoldChange}) > 1.5$  as the threshold for the identification of DEGs. Time course analysis for all the four time points were carried out to find genes that reacted in a condition-specific manner over time using DESeq2 package.

A total of 1.6 billion single-end reads consisting approximately in an average of 33 million reads for each cDNA library were obtained. Increased percentage of mapped reads was observed with days past inoculation that was well corroborated with the observed symptoms during disease progression over time. The highest number of DEGs for both the compatible (Topas-wild) and incompatible (Topas-Rlm2) were at 3 dpi. This number was reduced by around three-fold at 5dpi in both interactions and remained fairly similar level over



the next sampling times in Topas-Rlm2 plants. On the other hand, it increased steadily in Topas-wild plants to exceed by roughly three times the number of DEGS found in Topas-Rlm2 plants. Time series analysis showed high expression of Avr genes during biotrophic phase at 7 dpi and most of the highly expressed genes during the necrotrophic phase at 11 dpi were associated with molecular functions involved in catalase activity, hydrolases, CAZymes, peptidases, and transporters. Out of the 134 CSEPs, 35 genes showed no expression in both the compatible and incompatible host. A total of 28 genes showed their highest expression level at 7dpi in compatible interaction compared to incompatible interaction. Among the CSEPs, 15 genes showed high expression at 11 dpi compared to early growth stages and these effectors may have important role during necrotrophy.

In that study, we performed a comprehensive analysis of the *L. maculans* transcriptomes during compatible and incompatible interactions. Based on comparative transcriptome analyses, key genes were highlighted that dictate the interaction between canola and *L. maculans*. Among the genes of particular significance, our results have identified candidate effectors, TFs, CAZymes, peptidase and other pathogenesis related genes that are specifically involved in important stages of pathogenesis like biotrophy, switching of biotrophy to necrotrophy, and necrotrophy. The genes involved in compatible interactions but not in incompatible interaction offers insights into the potential mechanisms of pathogenesis in *L. maculans*-canola interactions.

We further performed a comprehensive analysis of the transcriptome of canola lines during compatible and incompatible interactions with *L. maculans*. This led to the observations that aquaporins appeared to have contrasting differential expression. We focused our subsequent analyses on those and extended the analyses to several Brassicaceae species for comparative purposes. Aquaporins (AQPs) are of vital importance in the cellular transport system of all living organisms. In our study, genome-wide identification, distribution, and characterization of AQPs were determined in *Arabidopsis lyrata*, *Capsella grandiflora*, *C. rubella*, *Eutrema salsugineum*, *Brassica rapa*, *B. oleracea*, and *B. napus* (canola). Classification and phylogeny of AQPs revealed the loss of XIPs and NIP-IIIs in all species. Characterization of distinctive AQP features showed a high level of conservation in spacing between NPA-domains, and selectivity filters. Interestingly, TIP3s were found to be highly expressed in developing seeds, suggesting their role in seed desiccation. Analysis of available RNA-seq data obtained under biotic and abiotic stresses led to the identification of AQPs involved in stress tolerance mechanisms in canola. In addition, analysis of the effect of ploidy level, and resulting gene dose effect performed with the different combinations of Brassica A and C genomes revealed that more than 70% of AQPs expression were dose-independent, thereby supporting their role in stress alleviation. This first in-depth characterization of Brassicaceae AQPs highlights transport mechanisms and related physiological processes that could be exploited in breeding programs of stress-tolerant cultivars.

#### 4. Issues

- Describe any challenges or concerns faced during the project. How were they overcome or how do you plan to overcome?



- Describe any potential changes to the work plan and the budget. How were or how will they be managed?

RNA-seq produces over billions of sequence reads that require extensive computational resources for effective analysis and storage of the many fold data generated during the process. To overcome the challenges, we have used Mammoth parallèle II managed by Calcul Québec and Compute Canada that provided us computational power and a temporary storage space. In addition, we have also purchased storage system to take all the backups from Mammoth server. A computational workbench CLC was also purchased for the RNA-seq data analysis.

### 5. Lessons Learned:

Describe the key lessons learned gained as a result of executing the project (e.g., a more efficient approach to performing a specific task for activity / project).

### 6. Future Related Opportunities:

Describe the next steps for the innovation items produced by the activity/project. Is additional research required? Is there potential for commercialization or adoption?

As a direct consequence of the results obtained in the project, and with the support of SaskCanola we submitted a proposal to the NSERC-Strategic program to investigate an original approach to reduce canola losses to diseases and stress through manipulation of AQPs. In spite of the highly competitive nature of the program (less than 20% success rate), our application was successful, and the project will be carried out through an annual budget of 185,000\$ over the next three years. This project represents a unique opportunity to develop new approaches to mitigate the effects of disease pressure and climate stress on canola.





AgrInnovation Program Stream B

**2017-18 Annual Performance Report**

Name of Recipient: Saskatchewan Canola Development Commission	
Project Title: Canola Disease Management Tools for the Prairies – Blackleg and Sclerotinia	
Project Number: AIP-P032	Period Covered by Report: 2017-04-01 to 2018-03-31
Activity #: 6 Name of Activity: <b>Durable blackleg resistance stewardship through knowledge of blackleg pathogen population, resistance genes and crop sequence towards the development of a cultivar rotation program in the Prairie Provinces</b>	Principal Investigator: Dilantha Fernando, University of Manitoba, Gary Peng – AAFC, Ralph Lange - Innotech

**1. Performance Measures.** See Annex A for an explanation of each measure.

Innovation Items	Results Achieved	Provide a description (2-3 paragraphs) for each item produced and describe its importance to the target group or sector. Explain any variance between results achieved and targets. Use plain language.
# of Intellectual property items flowing from the project	0	
# of new/improved products	0	
# of new/improved processes or systems	0	
# of new/improved practices	1	Growers may now select canola varieties based on major gene resistance. The understanding of the blackleg race profile across western Canada provided by this project will allow grower to utilize the R-gene (major gene) resistance labels which are now being provided. The R-gene labeling method has been adopted by WCC/RRC allowing commercial seed companies to label the R-genes utilized in their varieties. This provides growers information they need to make variety decisions while minimizing blackleg in their fields.
# of new varieties	0	
# of new/improved genetic materials	0	
# of new/ improved gene sequences	2	1. Obtained 1229 >1000 and >1000 <i>L. maculans</i> isolates from Manitoba, Alberta and Saskatoon respectively; More than 200 <i>L. biglobosa</i> isolates were isolated from Manitoba province.



Innovation Items	Results Achieved	Provide a description (2-3 paragraphs) for each item produced and describe its importance to the target group or sector. Explain any variance between results achieved and targets. Use plain language.
		2. Obtained two isolates with mutation in Chitin_bind 3 gene coding region using Crispr/cas9 system and obtained region sequence of chintin_bind3.
# of improved knowledge	4	<ol style="list-style-type: none"> <li>1. The frequency of <i>AvrLm3</i>, corresponding <i>R</i> gene of which is the most resistant resource used across Canada, displayed higher frequency in fields with longer crop rotation compared with no rotation. <i>AvrLm3</i> also had a significantly higher frequency in AB than SK or MB.</li> <li>2. In Saskatchewan a noticeable trend toward decreasing prevalence of <i>AvrLmS</i> was observed.</li> <li>3. Identification of callose synthase gene family in canola and investigation of their expression in different resistant material with infection by different isolates.</li> <li>4. Transformed <i>L. maculans</i> via <i>Agrobacterium</i> based transformation system</li> </ol>

Information Items	Results Achieved	Provide the complete citation for each item. Please see Annex A for examples.
# of peer reviewed publications	5	<ol style="list-style-type: none"> <li>1. Becker, Michael G; Zhang, Xuehua; Walker, Philip L; Wan, Joey C; Millar, Jenna L; Khan, Deirdre; Granger, Matthew J; Cavers, Jacob D; Chan, Ainsley C; Fernando, Dilantha WG (2017). Transcriptome analysis of the Brassica napus–Leptosphaeria maculans pathosystem identifies receptor, signaling and structural genes underlying plant resistance. <i>The Plant Journal</i>, 90(3), 573-586.</li> <li>2. Fernando, W. D., Zhang, X., Selin, C., Zou, Z., Liban, S. H., McLaren, D. L., ... &amp; Rong, L. (2018). A six-year investigation of the dynamics of avirulence allele profiles, blackleg incidence, and mating type alleles of <i>Leptosphaeria maculans</i> populations associated with canola crops in Manitoba, Canada. <i>Plant Disease</i>, 102(4), 790-798.</li> <li>3. Zou, Z., Liu, F., &amp; Fernando, W. D. (2018). Rapid detection of <i>Leptosphaeria maculans</i> avirulence gene <i>AvrLm4-7</i> conferring the avirulence/virulence specificity on Brassica napus using a tetra-primer ARMS-PCR. <i>European Journal of Plant Pathology</i>, 1-6.</li> <li>4. Zhang, X., &amp; Fernando, W. D. (2018). Insights into fighting against blackleg disease of Brassica napus in Canada. <i>Crop and Pasture Science</i>, 69(1), 40-47.</li> <li>5. Hubbard M, Peng G. (2018). The quantitative resistance to blackleg (<i>Leptosphaeria maculans</i>) in common Canadian canola cultivars is effective under both moderate- and high-temperature conditions. <i>Plant Pathol.</i> Doi: 10.1111 /ppa.12832.</li> </ol>



# of information items	1	F. LIU, C. SELIN, AND W. G. DILANTHA FERNANDO. Loss of function of chitin binding protein gene <i>CBPL</i> affects the pathogenicity of <i>Leptosphaeria maculans</i> the blackleg pathogen on canola. Poster. 94th Annual Conference, Canadian Phytopathological Society. June, 2017.
# of media reports		
# of information events	1	Fernando, D. Understanding durability, resistance and virulence in the canola-blackleg pathosystem. CanolaWeek. Saskatoon, SK. 2017/12/06.
		<b>Provide the # of attendees</b>
# of individuals attending information events	250	
		<b>Provide the # of attendees who intended to adopt new information or technology</b>
# of individuals attending information event who intend to adopt new innovation		
		<b>Provide the name, degree completed and date of completion</b>
# of persons who completed a M.Sc. or Ph.D. during project		

## 2. Executive Summary

The Executive summary contains two parts: Key highlights of activities and scientific results and Success story. Information may be used for internal and external communication purposes. Write for a general audience using plain language. Do not include sensitive or confidential information

**Key Highlights** - This section describes the key activities and final scientific results of an activity/project in such a way that readers can rapidly become acquainted with a large body of material without having to read it all. Include a brief statement of the problem(s), background information, concise analysis and main conclusions. Suggested length – maximum 1 page.

Blackleg caused by the fungus *Leptosphaeria maculans* (*L. maculans*) is a serious disease of canola in western Canada. R gene rotation can increase the durability of R genes. Mastering of Avr gene profiles will be helpful for R gene rotation. To obtain the avirulence (*Avr*) gene profiles in the prairies *L. maculans* isolates were tested for the presence of 11 *Avr* alleles (*AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4*, *AvrLm5*, *AvrLm6*, *AvrLm7*, *AvrLm9*, *AvrLm11*, *AvrLepR1*, *AvrLepR2* and *AvrLmS*) using a set of differentials combined with PCR amplification. The *Avr* profile in these targeted commercial canola fields is one of the key products of this project

In Manitoba *AvrLm4*, *AvrLm5*, *AvrLm6*, *AvrLm7* and *AvrLm11* displayed higher frequency with more than 80% in the pathogen population, while *AvrLm3* and *AvrLm9* showed frequency less than 5%. Race structures were analyzed based on the combinations of *Avr* genes, and a total of 82 races



were detected. Races carrying as many as ten, and as few as three, known *Avr* allele were detected. The race *Avr-2-4-5-6-7-11-S* ranked the most prevalent race with percentage as 24.19% during all races.

In Saskatchewan, blackleg occurred in each of the canola fields monitored, with disease incidence ranging from <10% to 78% and severity from trace to 2.5. Short crop rotation appeared to be one of the key factors for high levels of blackleg. *Avr* pictures varied depending on the field and year, but there was generally a trend of high *AvrLm6* and *AvrLm7*, and *AvrLm4* in some of the field years. *AvrLm1*, *AvrLm3*, *AvrLm9* and *AvrLep2* were often low while *AvrLm2*, *AvrLm5* and *AvrLep1* appeared variable. There was a noticeable trend of lower *AvrLm5* after 2016.

In contrast to Manitoba and Saskatchewan, *AvrLm1* and *AvrLm3* seemed quite common in Alberta. The frequency of other alleles showed some variation from site to site. We cannot comment on the frequency of some alleles, e.g. *Rlm6* and *Rlm7*, as we did not have access to a full set of differential *B. napus* lines. Pathogenic variability seems to occur at the field scale, as we cannot determine any stratification of pathotypes at the landscape scale. Since resistance among commercial *B. napus* cultivars is quite uniform (mostly *Rlm1* and *Rlm3*), variability among fields may be due either to genetic drift, or to response to selection pressure by other factors, such as quantitative resistance

Understanding the R-gene and the pathogen profiles in the prairies has helped the industry to launch the R-gene labeling system and rotation strategy.

**Success Story** - A success story presents a significant result or an important milestone achieved. It is intended to showcase achievements in applied research. Focus on research results, successful technology transfer, potential for pre-commercialization, and/or potential impact. A Success Story is not a progress report for each activity (suggested length 2 – 3 paragraphs).

A stronger understanding of blackleg pathogen population across Western Canada was gained which will help determine where to deploy specific varieties and R-genes to match the population. This will help to increase the durability of resistance genes and better manage the disease.

### 3. Objectives/Outcomes (technical language is acceptable for this section)

Provide a brief summary that includes introduction, objectives, approach/methodology, deliverables/outputs, results and discussion, and any Ph.D or Master students recruited to work on the project.

#### Introduction

Blackleg, caused by the fungus *Leptosphaeria maculans* (*L. maculans*), is one of the most devastating disease of canola worldwide. Crop rotation, disease-free seed, fungicides, seeds



treatments and resistant varieties were considered as effective strategies for controlling this disease (Kutcher et al., 2013). During these strategies, utilization of resistant varieties was recognized as the most effective and economic one. Both qualitative ( *R* gene) resistance and quantitative (adult plant resistance, APR) have been identified in Brassica species for against this pathogen. Strong association has been identified between seedling and adult plant resistance to *L. maculans* in *B. napus* (NEWMAN & Bailey, 1987, McNabb et al., 1993, Bansal et al., 1994). For qualitative resistance or race specific resistance, a total of 18 major R genes against *L. maculans* have been identified in Brassica species and 16 corresponding avirulent genes have been identified in *L. maculans* (Liban et al., 2016, Zhang et al., 2015). The *Avr* gene combinations of isolates can be obtained by analyzing response with differential varieties containing different R gene using cotyledon test or by PCR test with specific primers corresponding to cloned *Avr* genes. Moreover, long term using of varieties with single resistant resource will impose strong selection pressure on the *L. maculans* population in the fields, which can lead to evolution of corresponding avirulence genes, and ineffectiveness of R genes. Therefore, mastering of the blackleg avirulence gene profile will aid in understanding about *L. maculans* population in specific area, and will provide instructional information for growers. In addition, the information will provide guide for reasonable use of R gene, making their resistance more durable.

#### Objectives

1. To get knowledge on frequency of 12 *Avr* genes in *L. maculans* population;
2. To acquire the race structures of *L. maculans* population in the prairies
3. To master the trend of changes for each *Avr* genes especially for those of which the corresponding R genes were widely used

#### Methodology:

##### Fungal isolation and inoculum preparation

Isolation of *L. maculans* isolates was performed as described by Liban et al. (2016) with modification. Part of the infected stem was cut into small pieces and immersed into 30 % bleach solution. After 1 min surface sterilization treatment, the sterile pieces were placed on paper towel for drying. Then the dried stem pieces were moved onto V8 agar juice medium containing 0.0035% (w/v) streptomycin sulphate. And the plates were cultivated under continuous florescent light at room temperature (25 °C) for about 5 days. Single pycnidia was picked and grew on a new V8 agar plate with 0.0035% (w/v) streptomycin sulphate. After sporulation, 2.5 ml autoclaved sterile water was added onto the plates and pycnidia spores were harvested by scraping the surface of plate.

##### DNA extraction and PCR reaction

Genomic DNA of pycnidia spores were extracted according to CTAB method. *AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4-7*, *AvrLm5*, *AvrLm6* and *AvrLm11* were tested using PCR reaction. The identification of *AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4-7* and *AvrLm6* were performed with the



combination of pathogenicity test and PCR assay. For *AvrLm5* and *AvrLm11*, only PCR test were used for identifying *Avr* alleles since we don't have *corresponding* recognized varieties.

#### Pathogenicity test

Avirulence genotypes of *L. maculans* isolates were identified by interaction between *L. maculans* isolates and a set of *B. napus* cultivars. Eleven cultivars corresponding to 10 avirulence genes were seeded and grew for 7 days. The 11 differential cultivars can identify 10 avirulence genes including *AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4*, *AvrLm7*, *AvrLm9*, *AvrLepR1*, *AvrLepR2* and *AvrLmS*. Four lobes of the seven-day old seedlings were punched using a modified forceps. Ten microliters pycnidiospores suspension ( $2 \times 10^7$  spores per mL) were dripped onto the punched site. Seedlings were grown for 14 days after inoculation. Interaction phenotype were evaluated based on a rating scale of 0 to 9 (Delwiche & Williams, 1979). Six plants of each cultivar were used for the pathogenicity test of one isolate. The mean value of 24 rating scores were used for assay virulence/ avirulence interaction phenotype (virulent: IP=5-9; avirulent: IP=0-4.9). The avirulence profiles of each isolate were determined by each isolate–host.

#### Results and Discussion

Twelve known genes: *AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4*, *AvrLm5*, *AvrLm6*, *AvrLm7*, *AvrLm9*, *AvrLm11*, *AvrLepR1*, *AvrLepR2*, and *AvrLmS*, were tested for *L. maculans* isolates. In Manitoba fields, five *Avr* genes were detected in more than 80% of the population: *AvrLm4* (84.54%), *AvrLm5* (93.77%), *AvrLm6* (95.76%), *AvrLm7* (94.76%) and *AvrLm11* (90.02%). Two *Avr* genes were detected in less than 5% of the population: *AvrLm3* (4.74%), *AvrLm9* (4.24%). Other 5 *Avr* genes were detected from 11.22% to 76.14% in the population: *AvrLepR1* (11.22%), *AvrLepR2* (11.97%), *AvrLm1* (28.02%), *AvrLm2* (64.09%) and *AvrLmS* (76.14%).

In Saskatchewan, blackleg occurred in each of the canola fields monitored, with disease incidence ranging from <10% to 78% and severity from trace to 2.5. Short crop rotation appeared to be one of the key factors for high levels of blackleg. *Avr* pictures varied depending on the field and year, but there was generally a trend of high *AvrLm6* and *AvrLm7*, and *AvrLm4* was high in some of the field years. *AvrLm1*, *AvrLm3*, *AvrLm9* and *AvrLep2* were often low while *AvrLm2*, *AvrLmS* and *AvrLep1* appeared variable. There was a noticeable trend of lower *AvrLmS* after 2016

In contrast to Manitoba and Saskatchewan, *AvrLm1* and *AvrLm3* seemed quite common in Alberta. The frequency of other alleles showed some variation from site to site. We cannot comment on the frequency of some alleles, e.g. *Rlm6* and *Rlm7*, as we did not have access to a full set of differential *B. napus* lines. Pathogenic variability seems to occur at the field scale, as we cannot determine any stratification of pathotypes at the landscape scale. Since resistance among commercial *B. napus* cultivars is quite uniform (mostly *Rlm1* and *Rlm3*), variability among fields may be due either to genetic drift, or to response to selection pressure by other factors, such as quantitative resistance

Based on the Saskatchewan data, the blackleg impact on canola yield could not be determined since the yield data was not provided by all growers. In the cases where comparisons are possible,



longer rotations generally show lower levels of blackleg and a roughly 5 bu/ac yield increase in 2017. Most growers rotated canola cultivars, regardless of the crop rotation practices. None of the producers involved in the study reported the use of a foliar fungicide for blackleg control. Although severe cases of blackleg damage were uncommon in Saskatchewan, short rotations (continuous canola or a <2-year break) showed the higher risk of blackleg; there was likely sufficient inoculum in these fields and disease would occur when infection conditions materialize in early spring. At the same time, our data also showed that Rlm6, Rlm7 and Rlm4 (in some fields) could be highly effective for blackleg control. Once introduced, these new R genes are expected to perform well in minimizing the impact of blackleg on the prairies. Therefore, it is important to continue monitoring *L. maculans* population for effective use of new R genes or for R-gene rotation locally and regionally.

#### 4. Issues

- Describe any challenges or concerns faced during the project. How were they overcome or how do you plan to overcome?
- Describe any potential changes to the work plan and the budget. How were or how will they be managed?

For this project, we wanted to investigate the relationship between Avr gene frequency and crop rotation, but it was difficult to get all the growth history data from farmers. Moving forward, we are trying to cooperate with government and/or local agronomists to get these data.

#### 5. Lessons Learned:

Describe the key lessons learned gained as a result of executing the project (e.g., a more efficient approach to performing a specific task for activity / project).

#### 6. Future Related Opportunities:

Describe the next steps for the innovation items produced by the activity/project. Is additional research required? Is there potential for commercialization or adoption?



**AgrInnovation Program Stream B**

**2017-18 Annual Performance Report**

Name of Recipient: Saskatchewan Canola Development Commission	
Project Title: Canola Disease Management Tools for the Prairies – Blackleg and Sclerotinia	
Project Number: AIP-P032	Period Covered by Report: 2017-04-01 to 2018-03-31
Activity #: 7 Name of Activity: Investigating the Resistance (R-gene) durability of canola cultivars and the emergence of virulent blackleg isolates in farmers' fields	Principal Investigator: Dilantha Fernando, University of Manitoba

**1. Performance Measures.** See Annex A for an explanation of each measure.

Innovation Items	Results Achieved	Provide a description (2-3 paragraphs) for each item produced and describe its importance to the target group or sector. Explain any variance between results achieved and targets. Use plain language.
# of Intellectual property items flowing from the project	n/a	
# of new/improved products	n/a	
# of new/improved processes or systems		
# of new/improved practices	n/a	
# of new varieties	n/a	
# of new/improved genetic materials	n/a	
# of new/ improved gene sequences	1	Sanger sequencing was done for <i>L. maculans</i> gene <i>AvrLm2</i>
# of improved knowledge	5	<ol style="list-style-type: none"> <li>1. Since Rlm3 had less disease in 2017 compared to 2014, 2015 and 2016, cultivars carrying Rlm3 may be useful in rotation systems.</li> <li>2. An order of durability among the R genes tested; <i>LepR3</i> &gt; <i>LepR1</i> &gt; <i>Rlm2</i> &gt; <i>Rlm4</i> &gt; <i>Rlm3</i>, was recommended based on yield penalty with the increase of a unit of disease severity.</li> <li>3. Crop rotation may be effective in gaining back Avr-genes i.e. <i>AvrLm3</i> in this study.</li> <li>4. Shifting of <i>L. maculans</i> genes from avirulence to virulence was observed and can happen in a single cropping season.</li> <li>5. Point mutation was responsible for the shifting of avirulence to virulence.</li> </ol>





Information Items	Results Achieved	Provide the complete citation for each item. Please see Annex A for examples.
# of peer reviewed publications		The following two manuscripts will be submitted to a peer-reviewed journal; <ol style="list-style-type: none"> <li>1. Canola-wheat-canola a 2-year crop rotation increased the durability of <i>R</i> genes to <i>L. maculans</i>, a causal agent of blackleg disease in <i>Brassica</i> species</li> <li>2. Effect of <i>Brassica napus-Leptosphaeria maculans</i> interaction in the emergence of virulent isolates of <i>L. maculans</i>, a causal agent of blackleg disease in canola</li> </ol>
# of information items	n/a	
# of media reports	n/a	
# of information events	2	M. Harunur Rashid, Paula Parks, M. Hossein. Borhan and W.G. Dilantha Fernando. Canola-wheat-canola a 2-year crop rotation increased the durability of <i>R</i> genes to <i>L. maculans</i> , a causal agent of blackleg disease in <i>Brassica</i> species. CPS-MB-Regional Meeting, Winnipeg, MB Canada. November 6 <sup>th</sup> 2017.  M. Harunur Rashid, Paula Parks, M. Hossein. Borhan and W.G. Dilantha Fernando. Effect of <i>Brassica napus-Leptosphaeria maculans</i> interaction in the emergence of virulent isolates of <i>L. maculans</i> , a causal agent of blackleg disease in canola. 34 <sup>th</sup> Plant Science Graduate Student Association Symposium, University of Manitoba, Winnipeg MB Canada. March 18 <sup>th</sup> 2018.
		<b>Provide the # of attendees</b>
# of individuals attending information events	200	CPS MB Regional Meeting – 75 Graduate Student Symposium - 125
		<b>Provide the # of attendees who intended to adopt new information or technology</b>
# of individuals attending information event who intend to adopt new innovation	-	Unknown
		<b>Provide the name, degree completed and date of completion</b>
# of persons who completed a M.Sc. or Ph.D. during project		<b>Harunur Rashid</b> – started as a PhD student in January 2014. He submitted thesis to the Faculty of Graduate Studies in March 2018.

## 2. Executive Summary

The Executive summary contains two parts: Key highlights of activities and scientific results and Success story. Information may be used for internal and external communication purposes. Write for a general audience using plain language. Do not include sensitive or confidential information



**Key Highlights** - This section describes the key activities and final scientific results of an activity/project in such a way that readers can rapidly become acquainted with a large body of material without having to read it all. Include a brief statement of the problem(s), background information, concise analysis and main conclusions. Suggested length – maximum 1 page.

The use of cultivar rotation for limiting the blackleg pathogen adaptation to resistant genes is an emerging strategy that has had promising results in field trials in Australia (Marcroft et al, 2012). The University of Manitoba in collaboration with AAFC conducted a study to understand the blackleg pathogen population structure and the *R*-genes in Canadian Canola cultivars grown or developed by different seed companies, research institutes and universities. This information is important to recommend a cultivar rotation approach. However, before using this management technique effectively, we needed to fill knowledge gaps on the rate of pathogen adaptation to different resistance genes. Thus the first objective of this project is to assess which cultivar resistance genes are the most durable to disease pressure and to make recommendations on when and how often to rotate cultivars.

This first objective is studied by seeding plots with Topas-introgression lines (Topas-ILs) carrying different single dominant *R* genes (*Rlm* – resistance for *L. maculans*) and inoculating them with 90% avirulent plus 10% virulent isolates to the corresponding *R* gene and measuring the proportion of virulent isolates in the subsequent years. The rate of the population shifts to virulence is expected to vary between *Avr* alleles since there is a fitness cost associated with the loss of some *Avr*-genes (Huang et al, 2006). Establishing a timeframe for the majority of isolates to gain virulence will help us compare the relative durability of different *R* genes and make recommendations on when to rotate each cultivar depending on its *R* gene.

AAFC Saskatoon has recently developed ILs of *Brassica napus* by introgressing resistance genes into the susceptible DH line Topas (no *R* genes). These lines would allow us to limit the confusing effects of quantitative resistance and other variations from breeding backgrounds. The comparisons of growth and ratings of a single *Rlm* carrying Topas lines in response to blackleg would ideally be only affected by the resistance genes present and would provide a more reliable assessment of the durability of different *Rlm* genes.

The second objective of this research is to examine the potential of emergence of virulent isolates when a new cultivar without corresponding virulent isolates is introduced. *L. maculans* has shown the evolutionary ability to lose *Avr*-genes and gain virulence to *B. napus* as was seen with the emergence of new races in western Canada (Chen and Fernando, 2006; Kutcher et al, 2007). This is due to the high frequency of transposable elements in proximity to *Avr*-genes increasing the rate of mutation (Rouxel et al, 2011). In deploying a cultivar rotation strategy it is important to know the likelihood of breakdown of newly introduced *R* genes in the virgin fields.

The rate of mutation is expected to differ between different *Avr*-genes since those genes are located in different regions and clusters in the genome (Rouxel and Balesdent, 2005). This study is setup plots with ILs carrying different single dominant *R* gene (*Rlm* – resistance for *L. maculans*) and inoculated with 100% *Avr* isolates to the corresponding *R* gene. Susceptible line is used as a guard between canola and wheat to maintain disease pressure. We then monitored the field and recorded if and when virulent isolates emerge, recorded the number of generations required to produce virulent offspring, and compared results between *R* gene lines. All plots for both objectives were used in canola-wheat-canola 2-years rotation.

The recommended *R* genes rotation along with canola-wheat-canola 2-years crop rotation based on the *R*



gene durability and the nature of emergence of new races. It is also recommended that the R gene should be included alternatively from two different classes; group-1 (Rlm1, Rlm2, Rlm6 and LepR3) and group-2 (Rlm3, Rlm4, Rlm7, Rlm9 and LepR1), in a 2-year crop rotation to reduce the selection pressure on the fungal populations. Additionally, this study suggested an order of durability among the R genes tested; LepR3 > LepR1 > Rlm2 > Rlm4 > Rlm3, so that breeder can select more durable R genes for less disease. These data also suggested developing *B. napus* varieties with more effective R genes such as Rlm5, Rlm6, Rlm7 and Rlm11 as we have seen high frequency of the corresponding Avr genes in the *L. maculans* population.

**Success Story** - A success story presents a significant result or an important milestone achieved. It is intended to showcase achievements in applied research. Focus on research results, successful technology transfer, potential for pre-commercialization, and/or potential impact. A Success Story is not a progress report for each activity (suggested length 2 – 3 paragraphs).

The durability of some R genes used within commercial cultivars is now understood. This helped form the foundation for major gene resistance labels now used in blackleg resistance identification. Producers can now choose varieties with R genes that are durable to the specific blackleg races. Australia and Europe have used these labels to enable producers to choose the best gene that is most effective against the blackleg race present in their field. China has recognized that this was a gap in our risk mitigation plan and this research has provided background to develop the gene labelling model and provide the information to producers in an easy to use fashion. This will also play a significant role in reducing blackleg in the field in Canada.

### 3. Objectives/Outcomes (technical language is acceptable for this section)

Provide a brief summary that includes introduction, objectives, approach/methodology, deliverables/outputs, results and discussion, and any Ph.D or Master students recruited to work on the project.

This first objective of this study is to assess which R genes are most durable under disease pressure and from the collected data, make recommendations on when and how often to rotate R genes in the Canadian Prairies. The methodology involve setting up plots of ILs carrying different single resistance gene (R gene) and inoculating with 90% avirulent plus 10% virulent isolates to the corresponding R gene and measuring the proportion of virulent isolates in the subsequent years.

The second objective of this research is to examine the potential of emergence of virulent isolates when a new cultivar without corresponding virulent isolates is introduced. The methodology involves seeding ILs carrying different R gene and inoculate with 100% Avr isolates to the corresponding R gene. A patch of a susceptible line is used to maintain disease pressure in-between canola and wheat plot. The plots are monitored to record if and when virulent isolates emerge, record the number of generations required to produce virulent offspring, and compare results between the different R gene lines. All plots for both objectives are used the common canola-wheat-canola 2-years rotation.

A PhD student conducted project's work at the Ian N. Morrison Research Station at Carman and at the Department of Plant Science. The study is carried out using Topas-ILs developed by the AAFC Saskatoon to compare the durability of different blackleg resistance genes without the influence of host variation. The



ten plots were seeded with a susceptible cultivar Westar/Topas for the year 2013 to establish disease pressure for subsequent years by remaining stubble. The plots are separated by 55 meters intervals. Ten isolates were selected based on their race structures as inoculum for the plots. The necessary amount of inoculum was produced by the growing of *L. maculans* isolates on hundreds of agar plates in the lab. The inoculum was mixed at the required proportions for each plot and sprayed 3 times at a week intervals during the cotyledon stage to 2-4 leaf stage of canola growth (usually two weeks, three weeks, four weeks after seeding, respectively) for the year 2013 and 2014. But no inoculum was sprayed in the year 2015, 2016 and 2017 and allows them to infect from the guard rows. The plants were allowed to grow to maturity and disease incidence, and disease severity was recorded. At the end of the growing season in 2017, higher disease severity of 0.8 was found in the emergence trial from the cultivar harbouring the single *R* gene; *Rlm3*, and *Rlm4*. Infected stubbles from each plot were sampled and cultured. DNA extraction and PCR analyses indicated the *AvrLm4* completely disappeared in the isolates isolated from the stubble *Rlm4*. Similarly, *AvrLm2* was also disappeared in the isolates cultured from the stubble *Rlm2*. This is the indication of changes of *L. maculans* genes from avirulence to virulence.

Canola-wheat-canola two years crop rotation is effective in reducing the blackleg disease severity in canola. Crop rotation may effective in gaining back *Avr*-genes in *L. maculans* genome. Shifting of *L. maculans* genes from avirulence to virulence was also observed due to the accumulation of point mutation.

#### 4. Issues

- Describe any challenges or concerns faced during the project. How were they overcome or how do you plan to overcome?
- Describe any potential changes to the work plan and the budget. How were or how will they be managed?

Less than optimal disease pressure in the first year due to breaking new land for the trial site and non-optimal weather conditions. Late seeding in 2016 due to huge rain in the month of May. The high temperature and high moisture were also observed in 2017.

#### 5. Lessons Learned:

Describe the key lessons learned gained as a result of executing the project (e.g., a more efficient approach to performing a specific task for activity / project).

#### 6. Future Related Opportunities:

Describe the next steps for the innovation items produced by the activity/project. Is additional research required? Is there potential for commercialization or adoption?

Future research should assess the durability of any new blackleg *R* genes or stacked *R* gene combinations developed in breeding programs.



**AgrInnovation Program Stream B**

**2017-18 Annual Performance Report**

Name of Recipient: Saskatchewan Canola Development Commission	
Project Title: Canola Disease Management Tools for the Prairies – Blackleg and Sclerotinia	
Project Number: AIP-P032	Period Covered by Report: 2017-04-01 to 2018-03-31
Activity #: 8 Name of Activity: <b>Rapid field diagnostics of the blackleg pathogen races through the identification of pathogen avirulence (Avr) genes and the development of Avr-specific markers.</b>	Principal Investigator: M. Hossein Borhan, AAFC, Saskatoon Research Centre

**1. Performance Measures.** See Annex A for an explanation of each measure.

Innovation Items	Results Achieved	Provide a description (2-3 paragraphs) for each item produced and describe its importance to the target group or sector. Explain any variance between results achieved and targets. Use plain language.
# of Intellectual property items flowing from the project		
# of new/improved products		
# of new/improved processes or systems		
# of new/improved practices		
# of new varieties		
# of new/improved genetic materials	1	KASPar markers were developed for the recently cloned AvrLm9
# of new/ improved gene sequences		
# of improved knowledge		

Information Items	Results Achieved	Provide the complete citation for each item. Please see Annex A for examples.
# of peer reviewed publications	1	Ghanbarnia, K. , Ma, L. , Larkan, N. J., Haddadi, P. , Fernando, W. G. and Borhan, M. H. (2018), <i>Leptosphaeria maculans</i> AvrLm9: a new player in the game of hide and seek with AvrLm4-7. <i>Molecular Plant Pathology</i> . doi:10.1111/mpp.12658.



# of information items		
# of media reports		
# of information events		
		<b>Provide the # of attendees</b>
# of individuals attending information events		
		<b>Provide the # of attendees who intended to adopt new information or technology</b>
# of individuals attending information event who intend to adopt new innovation		
		<b>Provide the name, degree completed and date of completion</b>
# of persons who completed a M.Sc. or Ph.D. during project		

## 2. Executive Summary

The Executive summary contains two parts: Key highlights of activities and scientific results and Success story. Information may be used for internal and external communication purposes. Write for a general audience using plain language. Do not include sensitive or confidential information

**Key Highlights** - This section describes the key activities and final scientific results of an activity/project in such a way that readers can rapidly become acquainted with a large body of material without having to read it all. Include a brief statement of the problem(s), background information, concise analysis and main conclusions. Suggested length – maximum 1 page.

### Problem:

Genetic resistance to blackleg disease of canola (*Brassica napus*) caused by the fungus *L. maculans* is the most effective approach for controlling the disease. However, selection pressure causes emergence of new virulent *L. maculans* isolates. Emergence of new virulent isolates is due to mutation in Avr genes that enables *L. maculans* to avoid recognition of mutated Avr protein by matching receptor proteins (product of resistance genes) present in canola. Therefore for effective management of blackleg disease it is important to know the genotype of blackleg isolates within a field population. The goal of this research project is to develop marker-assisted genotyping as a fast and accurate approach for monitoring changes in *L. maculans* population in canola fields across western Canada.

### Background:

To date 16 resistance (R) genes (*Rlm1-11*, *RlmS*; *LepR1-4*) from Brassica have been identified that provide full immunity against *L. maculans* isolates with matching Avr genes (*AvrLm1-11*, *AvrLmS*; *AvrLepR1-4*). *AvrLm1*, *AvrLm4-7* and *AvrLm6* were cloned previously. *AvrLm11* and *AvrLm3* were recently reported. During the course of this project we cloned *AvrLm2* (1-9)



Allele polymorphism is routinely detected by PCR. An accurate and cost effective method of PCR based genotyping is Kompetitive Allele Specific PCR (KASP) method. KASP methodology uses two gene specific forward primers that differentiate alleles based on SNP, a common reverse primer and the KASP master mix that in addition to common PCR component also includes two fluorescent tags (HEX and FAM). If the allele is homozygous then one fluorescent signal will be generated and if it is a heterozygous mix, both signals will be generated.

Concise analysis and main conclusions:

During the course of this project and as we reported previously, we cloned *AvrLm2*. Here we report cloning of *AvrLm9* and progress toward identifying *AvrLep2*. We generated a cross between *L. maculans* isolates 00-100 (*AvrLm9*, *avrLm4-7*, *AvrLep2*) and v23.1.3 (*avrLm9*, *AvrLm4-7*, *avrLep2*). Segregation data identified suppression effect of a *L. maculans* gene on *AvrLm9* function. Complementation assay proved *AvrLm9* function is masked in the presence of *AvrLm4-7*. *AvrLm9* was mapped on scaffold 7 of V23.1.3 genome and proved to be an allele of previously reported *AvrLm5* (*AvrLmJ1*) (10).

**Success Story** - A success story presents a significant result or an important milestone achieved. It is intended to showcase achievements in applied research. Focus on research results, successful technology transfer, potential for pre-commercialization, and/or potential impact. A Success Story is not a progress report for each activity (suggested length 2 – 3 paragraphs).

China requested that Canada develop a rapid “in-field” test to identify blackleg races present on the canola stubble. The PCR biomarkers which were developed as part of this project were shared with public and private pathology labs across western Canada. Four labs now have the markers for commercial evaluation. When growers detect significant amounts of blackleg in their field they may now send stubble samples to pathology labs for genetic testing. The pathology lab will then provide the grower with information on the specific races of blackleg in their field. This will enable canola growers to make informed decisions about choosing the appropriate blackleg resistance in their canola variety that best matches the profile of the blackleg strain in the field. This is an important tool for managing blackleg in the field and addresses a concern of a major trading partner.

### 3. Objectives/Outcomes (technical language is acceptable for this section)

Provide a brief summary that includes introduction, objectives, approach/methodology, deliverables/outputs, results and discussion, and any Ph.D or Master students recruited to work on the project.

Introduction:

Blackleg, caused by the fungus *Leptosphaeria maculans* (Lm) is the most devastating disease of canola in Canada. Over the past decade blackleg on canola has been successfully controlled through the use of resistant cultivars. However selection pressure exerted by tight crop rotation and limited sources of resistance has resulted in the re-emergence of blackleg disease in Western Canada. The most recent example of severe blackleg disease on commercial resistant varieties of



canola was in Manitoba and Saskatchewan in 2012. Understanding the changing pathogen population structure in canola fields is the key factor in managing blackleg through the deployment of plants with resistance genes against prevalent avirulence (*Avr*) genes present in the pathogen population. The main objective of this proposal is to develop molecular markers as an efficient tool for genotyping and monitoring *L. maculans* populations in canola fields across Western Canada. These markers will be designed from a set of *Avr* genes matching major *L. maculans* resistance genes in canola cultivars. By knowing the prevalence of these *Avr* genes in the field populations of *L. maculans*, breeders will be able to develop appropriate cultivars with resistance genes targeted against prevalent pathogen races (based on *Avr* genes) on a regional basis.

#### Methodology:

A combination of map-based cloning and genome sequence comparison was applied to define the genomic location and clone the *Avr* gene. An F<sub>1</sub> population resulting from a cross between *L. maculans* isolates, v23.1.3 (*avrLm9*, *AvrLm4-7*, *avrLep2*) and 00-100 (*AvrLm9*, *avrLm4-7*, *AvrLep2*) were developed. The A mapping population, consisting of 73 F<sub>1</sub> progenies. KASP markers were developed across the *L. maculans* genome and position of *AvrLm9* was defined by linkage analysis. *AvrLm9* was expressed under its own promoter and its function was confirmed by restoring *AvrLm9* phenotype via transformation of the naturally virulent (*avrLm9*)

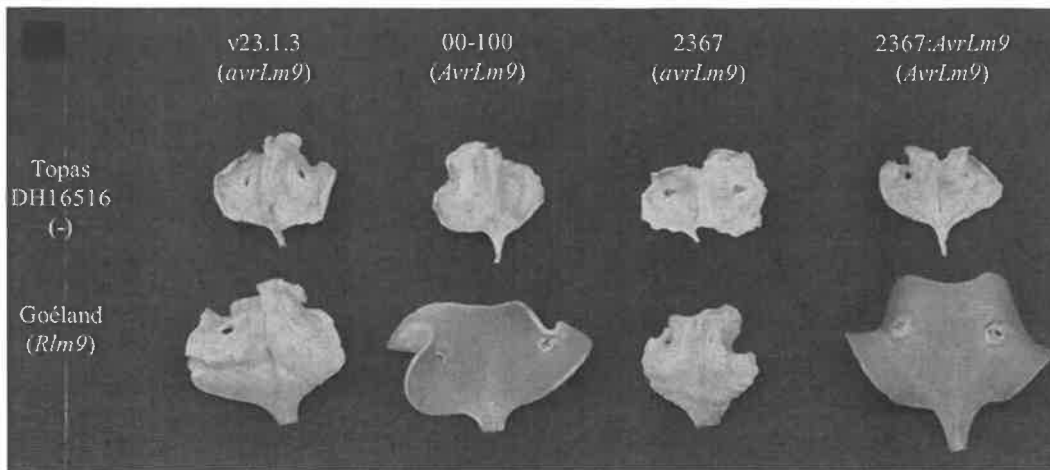
#### Deliverables/Outputs:

Cloning and developing KASP markers for *AvrLm9*

#### Results and discussion

A mapping population, consisting of 73 F<sub>1</sub> progenies from a cross between *L. maculans* isolates 00-100 (*AvrLm3*, *avrLm4-7*, *AvrLm9*) and v23.1.3 (*avrLm3*, *AvrLm4-7*, *avrLm9*), were tested on *B. napus* cv. Goéland (*Rlm9*). And *B. napus* cultivar Topas DH16516 as negative control. *Avr* to *avr* phenotype was observed as a 1: 3 segregation ratio. This indicated involvement of two genes in controlling *AvrLm9-Rlm9* interaction phenotype. By comparing the genome of 40 isolates and also the parent isolates for the cross-described above we noticed that whenever *AvrLm4-7* is present the isolate become virulent on *Rlm9* differential line. This supported masking effect of *AvrLm4-7* on the function of *AvrLm9*. We then mapped *AvrLm9* position selecting only progenies of the population that did not have *AvrLm4-7* allele. This resulted in placing *AvrLm9* on the scaffold 7 of v23.1.3 genome, co-segregating with the previously cloned *AvrLmJ1* that was recently proved to be *AvrLm5* (10). We cloned *AvrLm5* as a potential candidate for *AvrLm9* under the regulation of its native promoter and transferred it to the virulent *L. maculans* isolate 2367 (*avrLm3*, *avrLm4-7*, *avrLm9*). As shown in figure 1 transgenic 2367-*AvrLm9* restored the *Avr* phenotype when inoculated on Goéland (*Rlm9*). This proved that *AvrLm5* allele was *AvrLm9* and function of *AvrLm9* is masked when *AvrLm4-7* is present. We renamed *AvrLm9* as *AvrLm5-9* (11). We analysed the functional SNPs and developed KASP markers for genotyping *AvrLm5-9* allele.





**Figure 1: Proof of function of *AvrLm9* genomic clone driven by its native promoter.** *L. maculans* isolate 2367 is virulent on *B. napus* lines Goéland with *Rlm9* (lack of functional *AvrLm* 5-9). Upon transformation with *AvrLm* 5-9 constructs, recognition of 2367:*AvrLm* 5-9 transgenic line was restored when inoculated on Goéland cotyledons.

Using the same population we mapped the position of *AvrLep2* locating it on scaffold 3. A candidate predicted effector within *AvrLep2* genomic interval was identified as a most likely candidate for *AvrLep2*. Proof of function of this allele and its variant in other isolates is in progress.

#### References:

- 1- Fudal I, *et al.*, (2007) Heterochromatin-Like regions as ecological niches for avirulence genes in the *Leptosphaeria maculans* genome: map-based cloning of *AvrLm6*. *Mol Plant Microbe Interact* 20:459-470.
- 2- Ghanbarnia, K, *et al.*, (2012). Genetic mapping of *Leptosphaeria maculans* avirulence gene corresponding to the *LepR1* resistance gene of *Brassica napus*. *Theor. Appl. Genet.*, 124: 505-513.
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#### 4. Issues

- Describe any challenges or concerns faced during the project. How were they overcome or how do you plan to overcome?
- Describe any potential changes to the work plan and the budget. How were or how will they be managed?

NA

#### 5. Lessons Learned:

Describe the key lessons learned gained as a result of executing the project (e.g., a more efficient approach to performing a specific task for activity / project).

NA

#### 6. Future Related Opportunities:

Describe the next steps for the innovation items produced by the activity/project. Is additional research required? Is there potential for commercialization or adoption?



## Final Performance Report

This template is aimed to provide a summary of the performance results achieved against the targets identified in the work plans for the Contribution Agreement (CA) and the Collaborative Research and Development Agreement (CRDA) during the entire life of the activity/project.

Please write for a general audience using plain language. Do not include sensitive or confidential information.

Name of Recipient: Lone Buchwaldt	
Project Title: Canola Disease Management Tools for the Prairies – Blackleg and Sclerotinia	
Project Number: AIP- AIP-P032	Period Covered by Report: 2013-04-01 to 2018-03-31
Activity #: 10 Name of Activity: Characterization of defense genes underlying quantitative resistance loci (QRL) to Sclerotinia stem rot in Asian Brassica napus and transfer of resistance to Canadian spring type canola	Principal Investigator: Lone Buchwaldt, AAFC-Saskatoon. CRDA report for Lone Buchwaldt, Dwayne Hegedus, Isobel Parkin, Sally Vail, AAFC-Saskatoon.
Start Date (YYYY-MM-DD): 2013-04-01	End Date (YYYY-MM-DD): 2018-03-31

### 1. Summary of Performance Results for the entire life of the activity/project

**Targets:** Should be the sum of the targets that were set out in the work plan of the CA and in the Performance Measures Table for projects with a CRDA.

**Results Achieved:** Should be the sum of the results reported in all your Annual Performance Reports (APRs) including results achieved under the activities both in the CA and the CRDA.

**Explain any variance:** If the targets and the results achieved are different, provide a brief explanation using plain language. If there is no difference between the targets and the results achieved, leave it blank. Do not list each item of the results achieved here as they were already reported in the APRs. If a result was finalized but not included in any of the APRs, it can be reported here; however, you need to provide a brief description about the result and a brief explanation about why it was not reported in an APR.

Performance Measures	Targets	Results Achieved	Explain any variance between targets and results achieved. Use plain language.
# of Intellectual property items flowing from the project	<b>10:</b> Transfer of seed to canola breeding companies of <i>B. napus</i> germplasm lines with resistance to sclerotinia stem rot.	<i>Brassica napus</i> germplasm lines and pre-breeding lines are our performance measure. Seeds of some of them have been transferred to plant breeding companies, who will develop new canola varieties.	



	<p><b>2:</b> Two <i>B. napus</i> lines with resistance to sclerotinia stem rot, canola quality and good agronomy</p>	<p>A total of 10 canola breeding companies signed material transfer agreements (MTA) and received seed of four <i>B. napus</i> germplasm lines, PAK54, PAK93, DC21 and K22, which are resistant to sclerotinia stem rot, but do not have canola quality seed.</p> <p>Two pre-breeding lines were selected from a large population derived from a cross between the sclerotinia resistant line, PAK54, and AAFC's canola quality line, N99-508. The two pre-breeding lines have a combination of all desirable traits: sclerotinia resistance, canola quality, early flowering, high yield and good agronomy. The population is a resource for intercrossing of sister lines to combine these traits in more and increasingly better pre-breeding lines.</p>	<p>Activity 10.2: The number of canola quality lines with sclerotinia resistance is less than expected. This was partly due to a Post Doc leaving after one year for personal reasons. This coincided with AAFC's new hiring process which delayed hiring of a replacement. Subsequently, the new Post Doc went on 1-year maternity leave.</p>
<p># of new/improved products</p>			
<p># of new/improved processes or systems</p>	<p><b>4:</b> Four linkage maps showing the location of sclerotinia resistance in the <i>B. napus</i> genome and associated molecular markers</p>	<p><i>This item is an improved process because plant breeders can utilize more of the output data from DNA sequencing machines.</i></p> <p>Four linkage maps were developed for <i>B. napus</i> germplasm lines PAK54, PAK93, DC21 and K22. Molecular markers linked to sclerotinia resistance were identified. Plant breeders can more efficiently select breeding lines containing the sclerotinia trait based on of the presence/ absence of the molecular markers we have identified.</p> <p>A publication in 2018 will give the sector access to information on molecular markers and co-located defense genes conferring sclerotinia resistance.</p>	
<p># of new/improved practices</p>	<p><b>1:</b> A 'stem test' for evaluation of canola varieties with a claim of sclerotinia resistance.</p>	<p><i>This item is a new practice adopted by the canola industry sector.</i></p> <p>A so called 'stem test' was developed by the PI to screen <i>B. napus</i> plants for sclerotinia resistance. A group of pathologist and plant breeders, led by the PI, successfully evaluated the protocol at 33 locations. As a result, the Western Canadian Canola/</p>	<p>This activity was added during the project</p>



		Rapeseed Recommending Committee (WCC/RRC) adopted the 'stem test' protocol for evaluation of new canola varieties with a claim of sclerotinia resistance.	
# of new varieties			
# of new/improved genetic materials	<b>3:</b> A set of 17 sclerotinia isolates representative of the pathogen population in western Canada.	<i>Isolates of Sclerotinia sclerotiorum were characterized for genome variation and pathogenicity on B. napus and represent improved genetic material.</i>  Three canola breeding companies signed material transfer agreements (MTA) and received a set of 17 sclerotinia isolates representative of the genetic variation and pathogenicity of the fungal population in western Canada.	This tech transfer was added during the project
# of new/improved gene sequences	<b>6:</b> Six defense genes transformed into <i>B. napus</i> enhance sclerotinia resistance.	<i>Specific alleles of genes which contribute to sclerotinia resistance were identified and therefore represent improved gene sequences.</i>  Six defense genes were sequenced, cloned and transformed into <i>B. napus</i> . We showed that three genes in the lectin family, concanavalin, curculin and hevein, enhance sclerotinia resistance. Three other defense genes, WRKY33, annexin and O-methyl transferase, had smaller effect on resistance.	
# of improved knowledge			

2. New/Improved Products: Of the new/improved products developed and reported above during the project, which products have commercial potential? Which have been commercialized? And which have been used/adopted by the sector? Explain what stage each product is at and the impact on the sector.

The four *B. napus* germplasm lines, PAK54, PAK93, DC21 and K22, resistant to sclerotinia stem rot have commercial potential. A total of 10 canola breeding companies signed material transfer agreements (MTA) and received seed of these lines. Reports received in October 2017 from 7 plant breeders confirmed that at least 5 are actively developing canola varieties for western Canada using this material.

In 2015, the Western Canadian Canola/Rapeseed Recommending Committee (WCC/RRC) adopted the 'stem test' protocol for testing of canola varieties with a claim of sclerotinia resistance. Most canola breeders are now using the sclerotinia stem test to ensure potential new varieties meet the requirements of WCC/RRC.

So far, 3 canola breeding companies have signed material transfer agreements (MTA) for a set of 17 *Sclerotinia sclerotiorum* isolates. These isolates were characterized for genetic and pathogenic variation and



are therefore improved genetic material. They represent the pathogen population in western Canada. More companies will likely request these isolates once they have been published.

Impact on the sector: The new genetic material of *B. napus* is the best source of sclerotinia resistance available to plant breeders both in Canada and internationally. The linkage maps and molecular markers associated with sclerotinia resistance will improve marker-assisted-selection during variety development in the canola industry sector. The set of 17 well-characterized sclerotinia isolates are needed for development of canola varieties that hold up against the pathogen population in western Canada. It is expected that the first canola varieties with resistance derived from PAK54, PAK93 Dc21 or K22 will be available to growers in the next 2-3 years.

3. What is your target audience for sharing information about the results of your project? Describe your strategy and success in reaching this target audience.

The major target audience were plant breeders, plant pathologists and CEO's with private companies that develop canola varieties for western Canada. Data and information were presented every year primarily at Canola Industry meetings in Saskatoon and Winnipeg. In addition, there were meetings with each individual canola breeding company in Saskatoon.

Other audiences such as canola growers and extension staff in private and public sectors were reached at educational events (CanoLab), by articles in grower magazines and at field days (CanolaPalooza).

International scientists were reached during the International Rapeseed technical meeting in Switzerland in 2013, the International Rapeseed Congress in Saskatoon in 2015, and during a visit to Curtin University and University of Western Australia in Perth, Australia in 2017.



**AgrInnovation Program Stream B**

**2017-18 Annual Performance Report**

Name of Recipient: Saskatchewan Canola Development Commission	
Project Title: Canola Disease Management Tools for the Prairies – Blackleg and Sclerotinia	
Project Number: AIP- P032	Period Covered by Report: 2017-04-01 to 2018-03-31
Activity #: 11 Name of Activity: Resistance to <i>Sclerotinia sclerotiorum</i> necrosis inducing proteins in canola.	Principal Investigator: Dwayne Hegedus, AAFC, Saskatoon SK

**1. Performance Measures.** See Annex A for an explanation of each measure.

Innovation Items	Results Achieved	Provide a description (2-3 paragraphs) for each item produced and describe its importance to the target group or sector. Explain any variance between results achieved and targets. Use plain language.
# of Intellectual property items flowing from the project	0	
# of new/improved products	0	
# of new/improved processes or systems	0	
# of new/improved practices	0	
# of new varieties	0	
# of new/improved genetic materials	0	
# of new/ improved gene sequences	1	We completed the most involved analysis of the genes expressed by <i>S. sclerotiorum</i> during infection of a host plant, in this case canola, ever conducted. In total, over 40 million sequences were generated and the information published and deposited in a public database.
# of improved knowledge		



Information Items	Results Achieved	Provide the complete citation for each item. Please see Annex A for examples.
# of peer reviewed publications	2	Seifbarghi S, Borhan MH, Wei Y, Coutu C, Robinson S and Hegedus DD (2017) Deployment of the <i>Sclerotinia sclerotiorum</i> genome during infection of <i>Brassica napus</i> as revealed by RNA-Seq analysis. BMC Genomics 18:266 DOI 10.1186/s12864-017-3642-5.  Derbyshire M, Denton-Giles M, Hegedus DD, Seifbarghy S, Rollins J, van Kan J, Seidl MF, Faino L, Mbengue M, Navaud O, Raffaele S, Hammond-Kosack K, Heard S, Oliver R (2017) The complete genome sequence of the phytopathogenic fungus <i>Sclerotinia sclerotiorum</i> reveals insights into the genome architecture of broad host range pathogens. Gen. Biol. Evol. 9:593–618. (doi: 10.1093/gbe/evx030)
# of information items	0	
# of media reports	0	
# of information events	1	D. Hegedus. Sclerotinia stem rot in canola: Tales from the executioner’s handbook can lead to strategies for resistance. Canola Discovery Forum. Saskatoon (Dec. 6, 2017).
		<b>Provide the # of attendees</b>
# of individuals attending information events	200	
		<b>Provide the # of attendees who intended to adopt new information or technology</b>
# of individuals attending information event who intend to adopt new innovation		
		<b>Provide the name, degree completed and date of completion</b>
# of persons who completed a M.Sc. or Ph.D. during project		Shirin Siefbarghi (PhD student – anticipated completion in fall of 2018)

## 2. Executive Summary

The Executive summary contains two parts: Key highlights of activities and scientific results and Success story. Information may be used for internal and external communication purposes. Write for a general audience using plain language. Do not include sensitive or confidential information

**Key Highlights** - This section describes the key activities and final scientific results of an activity/





project in such a way that readers can rapidly become acquainted with a large body of material without having to read it all. Include a brief statement of the problem(s), background information, concise analysis and main conclusions. Suggested length – maximum 1 page.

### **The Problem**

*Sclerotinia sclerotiorum* causes disease on over 400 plant species; however, the level of resistance in virtually all major crops, in particular canola, is insufficient. Chemical control of the pathogen using fungicides is the only means currently available, but success depends on accurate prediction of pathogen load within the field and future weather conditions to ensure that coverage and timing of fungicide application coincides with release of fungal spores. At present, the only sources for Canadian breeders are winter-type lines from parts of Asia and the middle-East with partial resistance to stem rot resulting from the cumulative effects of several genes. This has made incorporation of the trait into spring-type canola difficult and has limited the development of stem rot resistant varieties by private sector canola breeders.

### **Background**

The best solution for Canadian farmers is to develop spring-type varieties with one or a few highly effective and robust stem rot resistance genes. This project contributes to this effort by providing both a more complete understanding of how the fungus causes stem rot in canola, and a highly targeted means to identify stem rot resistant lines. In this regard, we focused on how *S. sclerotiorum* causes the most noticeable and damaging aspect of disease in canola, namely, the necrotic lesions on the stem that lead to lodging and crop loss.

### **Concise Analysis and Main Conclusions**

1. We contributed to an international consortium that sequenced the *S. sclerotiorum* genome.
2. We catalogued the entire suite of genes expressed during each stage of the infection of canola that has led to a much better understanding of how *S. sclerotiorum* causes disease.
3. We identified several new proteins that are secreted by *S. sclerotiorum* that cause necrosis. We conducted experiments that will help to elucidate how they do this with the aim of using this information to develop plants that are impervious to their actions.
4. We began work to develop tools that will use these necrosis proteins to screen *B. napus* collections for lines that are more tolerant of or resistant to their effects.

**Success Story** - A success story presents a significant result or an important milestone achieved. It is intended to showcase achievements in applied research. Focus on research results, successful technology transfer, potential for pre-commercialization, and/or potential impact. A Success Story is not a progress report for each activity (suggested length 2 – 3 paragraphs).

1. The project contributed to an international collaboration between researchers in Australia (Curtin University), USA (University of Florida), Europe (INRA-CNRS France, Rothamsted Research UK, Wageningen University, The Netherlands) and Canada (AAFC Saskatoon) to sequence the *S. sclerotiorum* genome. We contributed the bulk of the RNA-Seq (gene expression) data for genome annotation, as well as the analysis of genes encoding effector proteins. The genome sequence was published and deposited in a public database. The genome sequence is now serving as a reference for a new international initiative to sequence the genomes a global collection of *S. sclerotiorum* isolates from a multitude of crops. This will provide important information regarding host specificity, host adaptation, and *S. sclerotiorum* populations from around the world.
2. The project catalogued, for the first time, the entire suite of genes expressed by *S. sclerotiorum*



during the infection of canola. This contributed immensely to our understanding of how this pathogen causes disease, including the identification of *S. sclerotiorum* proteins that cause the necrotic symptoms associated with this disease. We are aware that this information is assisting Canadian researchers (M. Belmonte and S. Whyard, University of Manitoba) in developing exciting new tools to disrupt the expression of genes vital for *S. sclerotiorum* infection based on RNA interference (RNAi). In the interests of efficiency, we are coordinating efforts with this group. In an allied project, we have generated *B. napus* lines expressing RNAi constructs that target two chitin synthase genes important for *S. sclerotinia* growth. These are to be examined for *S. sclerotiorum* resistance in 2018. Finally, we are collaborating with researchers in Australia and France in a new, but related, area of research based on a recent discovery that fungal plant pathogens produce small RNA molecules that are transferred to the host and interfere with the expression of genes involved in defense against the pathogen. We have generated most of the small RNA data from *S. sclerotiorum* infecting canola with the software applications for small RNA identification tailored to *S. sclerotiorum* being developed by our collaborators. These examples clearly illustrate how a relatively small investment (this project funded only a single graduate student) can be used to leverage additional resources, many-fold, by simply being collegial and involved to some degree in an area of research.

### **3. Objectives/Outcomes (technical language is acceptable for this section)**

Provide a brief summary that includes introduction, objectives, approach/methodology, deliverables/outputs, results and discussion, and any Ph.D or Master students recruited to work on the project.

#### **Introduction**

This project builds on work conducted by a former Ph. D student (Zafer Dallal-Bashi) who was supported in part by a scholarship from SaskCanola. The student discovered two types of proteins that are released by *S. sclerotiorum* that cause host plant tissue to undergo necrosis or die. This is the major disease symptom leading to stem collapse. The new project employed another graduate student (Shirin Siefbarghi) who identified the entire suite of proteins produced by this pathogen during infection of canola, tested these proteins for their ability to cause necrosis in plants and conducted experiments to determine how they function. The intent is to use proteins that contribute to necrosis to identify *B. napus* lines that are less susceptible to their effects.

#### **Objectives**

The main objectives of this research were to identify all of the proteins secreted by *S. sclerotiorum* that contribute to necrosis and disease symptoms, and to develop a method to screen *B. napus* lines for resistance/tolerance to their effects. The project was divided into three distinct phases that are described and reported upon below.

#### **Progress on Objectives**

**Phase I: Informatics Analysis to Identify Candidate *S. sclerotiorum* Necrosis Inducing Proteins.**

**Status: Completed.**

In January 2014, we recruited a Ph.D. student (Shirin Seifbarghi) through the Dept. of Biology at



the University of Saskatchewan. Ms. Seifbarghi already had a M.Sc. degree in Plant Pathology and is being co-supervised by Dr. Yangdou Wei.

The original draft genome of *S. sclerotiorum* was published by Amselem et al., in 2011. As an extension of this SaskCanola project, we contributed to an international consortium that used long read PacBio sequencing to complete the sequencing and annotation of the *S. sclerotiorum* genome (Derbyshire et al., 2017). The final complete genome contains 38,806,497 base pairs (bp) distributed across 16 chromosomes and with 11,130 non-ambiguous genes.

We were interested in genes that encode secreted proteins since necrosis proteins must be released from the fungus to exert their effect on the host plant. Potential necrosis proteins secreted from *S. sclerotiorum* were identified using computational analysis of genomic and transcriptomic (gene expression) information. The initial bioinformatic analysis was conducted using the original genome sequence and predicted the genes that encode proteins with a signal peptide (i.e. potentially secreted) using SignalP 4.1; approximately 900 such genes were identified. Further annotation of these genes using BLAST revealed that many of them had known functions and could be excluded from further analysis. Finally, approximately 100 candidate genes were selected that encoded proteins with features associated with necrosis-inducing proteins, such as being small, secreted, cysteine-rich, but without transmembrane domains, a GPI-anchor site or a vacuole/organelle-targeting motif.

Secondly, a replicated gene expression study (RNA-Seq) was conducted to characterize *S. sclerotiorum* genes expressed during infection of *B. napus*, with particular focus on the early infection stages. This was published as a stand-alone paper as it was the first ever to describe how the entire suite of *S. sclerotiorum* genes are expressed throughout the course of infection on any host (Seifbarghi et al., 2017). The study also provided evidence to substantiate a suspected biotrophic phase that occurs soon after host penetration, but prior to spread of the pathogen throughout the host. In the future, it would be highly interesting to study this stage more thoroughly as the pathogen is likely deploying factors that compromise host defenses and allow it to accumulate fungal biomass prior to rapid progression throughout the host. It will be at these early stages of the infection (penetration and biotrophy) where resistance is most likely to be achieved.

The expression pattern of the 100 candidate genes was determined based our gene expression data. This analysis returned 26 candidate genes that were highly expressed. These were subjected to *in planta* expression to determine if they are capable of causing necrotic lesion formation (Phase II).

As noted above, the project contributed to an international collaboration to sequence the *S. sclerotiorum* genome. We contributed the RNA-Seq (gene expression) data from this project for genome annotation, as well as the analysis of genes encoding effector proteins. In addition, we initiated a new collaboration with researchers in Australia and France to identify *S. sclerotiorum* small RNA molecules that may be transferred to the host and interfere with the expression of genes involved in defense against the pathogen. We generated small RNA data from the existing *S. sclerotiorum* infection on canola time course samples with the software applications for small RNA identification tailored to *S. sclerotiorum* being developed by our collaborators.

## **Phase II: Characterization of Candidate Necrosis Inducing Proteins.**

**Status: Completed.**



Bioinformatic and transcriptomic analysis of the proteins encoded by the *S. sclerotiorum* genome yielded 26 candidates that had properties indicative of effector proteins that contribute in some manner to the infection process. Initially, 8 candidate necrosis proteins with early and high expression levels were tested using an *Agrobacterium*-mediated *in planta* expression system. These genes were cloned using the Gateway® system into the pEG100 binary vector and were transformed into *Agrobacterium tumefaciens* GV3101. The bacteria expressing the candidate necrosis proteins were then infiltrated into leaves with positive and negative controls on the same leaf. 5 of the 8 candidates were found to cause necrotic symptoms in these tests, thus validating the informatics analysis. In this reporting period, the 18 remaining genes that were expressed at the later stages of infection were tested and several additional novel necrosis-inducing proteins were identified. Constructs were also generated for all of the 26 candidates to fuse the proteins to the green fluorescent protein (GFP) to help identify the targets of these effector proteins; these were also infiltrated into the host plant. This allowed the use of epifluorescence and confocal microscopy to see where in the host plant/cell the effector proteins go, and provided information as to what their targets may be and how they function. We are currently preparing a manuscript for publication that describes the informatics exercise and screening effort that identified these new *S. sclerotiorum* necrosis proteins.

One of the proteins from the initial group of 8 candidates exhibited an especially strong necrotizing activity under both light and dark conditions with activity equal to or greater than that of the *S. sclerotiorum* necrosis and ethylene producing proteins (NEP1 and NEP2) that we identified in an earlier project. The gene encoding this new type of necrosis protein is found only in *S. sclerotiorum* and in the closely related plant pathogen *Botrytis cinerea*. A protein identified in the second group of 18 candidates was also found to exhibit strong and consistent necrotizing activity. Detailed characterization of these novel necrosis proteins is continuing as part of the final chapter of the graduate student's thesis project that we anticipate will be completed by the end of 2018.

### **Phase III: Testing Candidate Necrosis Inducing Proteins on *B. napus*.**

#### **Status: In Progress.**

In the final phase of the project, tools to use necrosis proteins to screen a *B. napus* diversity collection for lines that are more resistant to their effects were to be developed. Targeting the most important attribute of this disease (the formation of necrotic lesions) should make it easier to identify lines with resistance to stem rot that can be incorporated into breeding programs. The *Agrobacterium*-based infiltration system used to identify necrosis proteins works well in *Nicotiana* species, but not as well in *B. napus*. Therefore, we expressed the most potent necrosis protein from the original set described above in bacteria (*Escherichia coli*) and yeast (*Pichia pastoris*) so that we could apply it directly to the plant, as has been done with necrosis proteins from other plant pathogens. Unfortunately, the purified protein expressed in these systems did not induce necrotic lesions. This may be due to improper folding or processing of the proteins in a heterologous system. We have undertaken work to express this protein and several other necrosis proteins in an engineered strain of *E. coli* capable of handling proteins that are rich in cysteine residues that require extensive folding for activity. The set will include the *S. sclerotiorum* NEPs described previously, the *S. sclerotiorum* ortholog of the necrosis-inducing xylanase described in *B. cinerea*, as well as 5 other *S. sclerotiorum* necrosis proteins. This work will extend beyond the end of this project and may be funded as part of the Canola Cluster.

## **4. Issues**



- Describe any challenges or concerns faced during the project. How were they overcome or how do you plan to overcome?
- Describe any potential changes to the work plan and the budget. How were or how will they be managed?

Please refer to the report on Phase III: Testing Candidate Necrosis Inducing Proteins on *B. napus*. In this regard, I was fortunate to have a technician assigned to my laboratory that was able to assist the graduate student in moving forward with the practical application of her work. I am hoping that AAFC management will agree to continue with the current assignment of technical resources so that we can make some more progress on this front if the funding is renewed.

#### 5. Lessons Learned:

Describe the key lessons learned gained as a result of executing the project (e.g., a more efficient approach to performing a specific task for activity / project).

As noted under Success Stories, this project clearly illustrates how even a small investment can be used to leverage additional resources, many-fold in this case, by providing opportunities to be involved in larger national and international consortia/collaborations to expand an area of research.

#### 6. Future Related Opportunities:

Describe the next steps for the innovation items produced by the activity/project. Is additional research required? Is there potential for commercialization or adoption?

We are collaborating with researchers in Australia (Curtin University) and France (INRA) to explore inter-kingdom transfer of small RNA from *S. sclerotiorum* to host plants. The hypothesis is that *S. sclerotiorum* produces small RNAs that engage the host plant RNA interference system and reduce the expression of host genes involved in aspects of defence/immunity. This has been demonstrated in fungal pathogens closely related to *S. sclerotiorum*. We and our collaborators have generated and pooled our small RNA sequence data, with researchers in Australia developing software applications for small RNA identification tailored to *S. sclerotiorum*. This is very new area of research for plant pathogenic fungi.

In the final phase of the project, tools to use necrosis proteins to screen a *B. napus* diversity collection for lines that are more resistant to their effects were to be developed. Targeting the most important attribute of this disease (the formation of necrotic lesions) should make it easier to identify lines with resistance to stem rot that can be incorporated into breeding programs. We have undertaken work to express a broad set of *S. sclerotiorum* necrosis proteins including the *S. sclerotiorum* NEPs described previously, a necrosis-inducing xylanase, as well as 5 of the most potent newly discovered necrosis proteins. This work will extend beyond the end of this project and a proposal for funding has been submitted to the Canola Cluster.

We participate and contribute to the international effort to assess the global diversity of *S. sclerotiorum* through genome sequencing. My colleague, Dr. Lone Buchwaldt, has already contributed isolates representing contemporary *S. sclerotiorum* populations on the Canadian prairies to this effort.