Government of Saskatchewan
Ministry of Agriculture

ADF Project Final Report Format

1. Project title and ADF file number.

20100126 MIDAS#024018 Final Phase Research to Improve "Hairy Canola' trait in *Brassica napus*

2. Name of the Principal Investigator and contact information.

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*The project was originally awarded to Drs. Margaret Gruber (principal investigator) and Juliana Soroka. Dr. Gruber became ill in mid-2014 and I was subsequently asked by AAFC management to assume responsibility for the project. Drs. Gruber and Soroka are now retired.

3. Name of the collaborators and contact information.

The project was co-funded by SaskCanola and a copy of this final report will be submitted to them.

4. Abstract/ Summary: *This must include project objectives, results, and conclusions for use in publications and in the Ministry database. Maximum of 300 words in lay language.*

Flea beetles are the most economically damaging pest of canola since they feed voraciously on young seedlings as they emerge from the soil in spring. At present, there are no useful *Brassica napus* (canola) lines available to breed for flea beetle resistance, and de-registration has restricted the use of several major insecticides. Approximately, $30M per year in insecticides are used to control flea beetles, but they still generate crop losses of $200M to $300M even with annual insecticide applications. Pesticide use of this magnitude may also contribute to producer health problems and has a negative impact on environmental health through pesticide contamination of soil and rural water systems. The project sought to develop canola lines with superior resistance to the crucifer flea beetle by enhancing a natural insect control system. Some plants produce hairs (trichomes) on their leaves which are a barrier to insects, reduce water loss in seedlings during drought and increase tolerance to freezing by providing a warmer microclimate around trichome-bearing tissues. Trichomes may also provide chemical protection against herbivores and diseases. Project researchers had previously developed a 'hairy' canola by introducing a gene from a trichome-bearing distant relative of canola. The objective of the current project was to identify lines of *Brassica napus* or its close relatives with the capacity to produce trichomes and make them, or the genes responsible for trichome production, available to the canola breeding community. The project identified several key genes that regulate trichome production in moderately hairy lines of Argentine (*Brassica napus*) and Polish (*Brassica rapa*) canola. The project also identified an extremely hairy line of *Brassica villosa* (related to the vegetable Brassicas) as a valuable resource to access trichome-related traits.

5. Introduction: *Brief project background and rationale.*

The project sought to develop superior *Brassica napus* (canola) breeding germplasm with resistance to the crucifer flea beetle by developing new hairy canola lines with the most optimal combinations

of trichome (plant hair) coverage genes. The project set to improve upon existing canola breeding germplasm, none of which has resistance to flea beetles. Trichomes also have the potential to reduce water loss in seedlings during drought. The project developed and advanced new lines with combinations of knock-down and over-expression constructs for 5 trichome regulatory genes required for dense trichome coverage, flea beetle resistance, and healthy plant growth. A few of these lines had already been developed with funding provided earlier by the canola industry or by Agriculture Canada, but the remaining combinations needed to be developed to find the most superior canola lines with dense trichome coverage, flea beetle resistance, and healthy growth characteristics. In addition, the project examined the gene expression patterns in hairy lines of *Brassica napus*, *Brassica rapa* and *Brassica villosa* to identify genes/alleles associated with trichome production.

6. Methodology: *Include approaches, experimental design, methodology, materials, sites, etc.*

The detailed methodology is outlined in the original proposal of which I have only a hard copy. I have made every attempt to provide sufficient detail in the progress report below and in the associated publications at the end of the report so the reader may understand the rationale, experimental design and methodology.

7. Research accomplishments: *(Describe progress towards meeting objectives. Please use revised objectives if Ministry-approved revisions have been made to original objectives.)*

Objectives	**Progress**
1. Development of new tester lines.	1. GL3 orthologs from other species GL3 is part of a regulatory complex that controls aspects of plant development, including trichome formation and anthocyanin production. Mutation of *GL3* leads to the glabrous (hairless) phenotype in Arabidopsis. Previously, we found that transformation of *B. napus* cv. Westar with the Arabidopsis *AtGL3* genomic sequence produced lines with abundant trichomes. To test if post-transcription alternative splicing caused the dis-function of *B. napus GL3*, we transformed *B. napus* plants with an *AtGL3* cDNA construct (35S:: AtGL3 cDNA). These lines had comparable levels of trichomes to the plants that expressed the *AtGL3* genomic DNA. The phenotype of the *AtGL3* cDNA plants, in terms of growth, leaf development and flower production, were comparable with plants that expressed the *AtGL3* genomic DNA. We also cloned the *B. rapa BrGL3* gene (which was most similar to *B. napus GL3-1*) from a moderately (M) hairy line and the Arabidopsis *AtGL3* cDNA (as a positive control gene) and developed binary constructs for each of these two genes under the control of either the constitutive *35S* promoter or a leaf specific *CER6* promoter. The *CER6* promoter is specific to leaf epidermal tissues and was intended to diminish the negative effect of *AtGL3* over-expression on the growth and development of transgenic *B. napus*. The *CER6::AtGL3* cDNA lines did not produce high levels of trichomes as observed by *35S::AtGL3* cDNA lines; however, the *35S::BrGL3-M* lines had 20-40% more trichomes than Westar (Figure 1). The *B. rapa GL3* was also cloned from an accession with high amounts of trichomes and an over-expression construct (*35S::BrGL3-G*) was

introduced into Westar. These transgenic lines had an 80% increase in trichomes compared to Westar, but the amount of trichomes was still less than *35S::AtGL3* lines. These experiments indicate that the *B. rapa and B. napus GL3* encode poorly functioning GL3 proteins. The two *B. rapa* GL3 proteins from the hairy lines have 5 amino acid differences (A242E, T247H, L339F, G378K and T535I).

To further investigate why the *AtGL3* induces abundant trichome production when the *B. napus* or *B. rapa GL3* orthologs do not, we generated three chimaeric *GL3* genes that replaced parts of the non-functional *B. napus GL3* gene with parts of the functional Arabidopsis *GL3* gene. In the first construct, domain 1 of the *BnGL3-1* (amino acids 1- 212) involved in interaction of GL3 with MYB proteins including GL1 (positive regulator), CPC and TRY (negative regulators) of trichome formation were replaced with first domain of *AtGL3*. In the second construct, the domain of *BnGL3-1* (amino acids 212-401) involved in interaction of GL3 with TTG1 was replaced by corresponding domain of *AtGL3*. In the third construct, the region encoding the carboxy terminal domain of BnGL3-1 containing the bHLH motif (ca. 200 amino acids) which mediates homodimer interactions was replaced by the region encoding the carboxy terminal domain of AtGL3. When over-expressed in *B. napus* cv. Westar under the direction of the *35S* promoter, all of the chimeric constructs improved trichome production to some degree, but not to the level observed with *AtGL3* (Figure 2).

Individual *B. napus GL3* genes from naturally hairy and non-hairy canola accessions (see Objective 2 below) were assessed for structural differences or expression pattern variation relative to the presence or absence of trichomes on these accessions. As well, we compared the structures of *GL3* and *EGL3* genes from *B. rapa* (mainly hairy species), *B. oleracea* (mainly non-hairy species), and *B. villosa* (extremely hairy species). Large structural variations occurred between the *EGL3* orthologues between hairy and non-hairy canola lines and the other Brassica species. From this gene structure data, we could not determine sequence differences that could lead to production or absence of trichomes in some Brassica species. However, one *BnGL3-1* homeologue in *B. napus* Westar was more similar in structure to the *BrGL3* gene in hairy *B. rapa*. In contrast, the *BnGL3-2* homeologue in *B. napus* Westar was more similar to the *BoGL3* gene in non-hairy *B. oleracea*, except that the protein it encoded was 135 amino acids shorter at the amino-terminus than BoGL3. However, the homodomains in the carboxy-terminus of both BnGL3-2 and BoGL3 were the same.

PCR-based expression analysis of young leaf cDNA from a mildly hairy accession of a natural *B. napus* line with lots of trichomes (113136) amplified only the *BnGL3-1* allele, whereas an accession with fewer trichomes (113160) and Westar (with very few trichomes) expressed both *BnGL3* gene homeologs. In contrast, only the *BnGL3-2* gene was expressed in leaves of two naturally non-hairy *B. napus*

accessions (101864 and 113131). From these expression data, we concluded that the *BnGL3-1* gene can lead to trichome production if the effect is not disrupted by expression of the *BnGL3-2* gene.

Brassica villosa is a wild species related to canola, but having extremely dense trichome patterning over the whole plant (Figure 3). The *BvGL3* gene was also cloned from an extremely hairy *B. villosa* line and introduced into *B. napus*. Transgenic lines have been generated and these will be phenotyped in the next few months.

2. Other trichome regulatory genes

RNA sequencing indicated much higher expression of the *GL2* and *TTG1* regulatory genes in *B. villosa* leaves compared with expression levels of *GL1* and *EGL3* genes in either *B. villosa* or the reference genome species, glabrous *B. oleracea* (see objective 2 below). RNA sequencing and qPCR also revealed an unusual expression pattern for the negative regulator *TRY* and *CPC* genes, which were much more highly expressed in trichome-rich *B. villosa* leaves than in glabrous *B. oleracea* leaves. The *B. villosa TRY* expression pattern also contrasted with *TRY* expression patterns in two diploid Brassica species, and with the Arabidopsis model for expression of negative regulators of trichome development. Further unique sequence polymorphisms, protein characteristics, and gene evolution studies highlighted specific amino acids in GL1 and GL2 sequences that distinguished glabrous species from hairy species and several variants that were specific for each *B. villosa* gene. Positive selection was observed for GL1 between hairy and non-hairy plants, and as expected the origin of the four expressed positive trichome regulatory genes in *B. villosa* was predicted to be from *B. oleracea*. In particular, the unpredicted expression patterns for *TRY* and *CPC* in *B. villosa* suggested additional characterization is needed to determine the function of the expanded families of trichome regulatory genes in more complex polyploid species within the Brassicaceae. The *B. villosa* trichome regulatory genes (*BvGL1*, *BvGL2*, *BvTTG1*, and *BvTRY*) were tested to determine whether they function differently from homologous genes in the non-hairy *B. napus* and Arabidopsis and to develop transgenic hairy canola with genes and promoters with no IP restrictions. Transgenic lines are under development.

Three RNAi knockdown constructs containing the *SPINDLY, HDG,* and *TB-LIKE-45* negative regulatory genes under the control of the *CER6* leaf-specific promoter were developed and introduced into *B. napus* to develop additional tester lines. Information regarding how and why these genes were selected is provided under objective 2 below. None of the *HDG* lines showed enhanced leaf trichome density; however, *SPINDLY* and *TB-LIKE-45* RNAi knockdown lines showed between 30-80 percent increase in trichomes on true leaves (Figure 4).

	3. Natural *hairy B. napus* lines Two-way crosses were developed between the two mildly hairy natural *B. napus* lines (#113136 and #113160) to determine whether natural (non-transgenic) plants with higher trichome density compared to the parents could be developed. The first generation after crossing didn't produce lines with higher amounts of trichomes compared to the parents. F1 plants were left to self-pollinate to see if the F2 generation had higher amounts of trichomes or they were back-crossed to the parent lines. Self-pollination of 113136 alone for four generations yielded lines with increased trichome production with no apparent effect on growth rate or productivity as seen in the *AtGL3* over-expression lines. Several of these were converted to doubled haploid lines to fix the trait (Figure 5). Selected doubled haploid lines have higher trichrome production compared to the parents and higher growth rate compare to the Westar.
2. Diversity of expressed genes and transcript abundance in trichome-rich and trichome-barren Brassica lines in the A, B, C and AC genomes.	This sub-objective was undertaken with the aim of finding lines with differentially expressed trichome genes and using this knowledge towards the development of non-GMO hairy canola. Initially, a natural genetic diversity study was conducted in which 995 accessions were assessed for growth and trichomes at the seedling stage and phenotyped for trichome location, trichome density, trichome patterning (even, clustered), and trichome size (short, medium, long). The phenotyped accessions included natural germplasm within all three diploid Brassica species and one tetraploid Brassica species: genome AA (represented by 652 lines of *B. rapa*), genome BB (represented by 21 lines of *B. nigra*), genome CC (represented by 26 lines of *B. oleracea* and 1 line of *B. villosa*), as well as the complex AACC genome (represented by 296 lines of *B. napus*). In an effort to understand the relationship between gene expression levels of all 236 trichome genes (including all trichome initiation and repression genes) and how they relate to different trichome phenotypes in the Brassica family, the transcriptome of a subset of representative accessions was analyzed by RNA sequencing of cotyledons and first true leaves, including 1 extremely hairy *B. villosa* line, 4 *B. rapa* lines (2 hairy and 2 non-hairy), 4 *B. napus* lines (2 mildly hairy and 2 non-hairy), a control non-hairy *B. napus* line cv. Westar line, 4 *B. nigra* lines, 5 *B. oleracea* lines, and 2 transgenic very hairy *B. napus* lines (Hairy 1 expressing the AtGL3+ expression construct in a Westar background, and Hairy 2 K-5-8 expressing the AtGL3+::BnTTG1-knockdown RNAi construct in a Westar background). We compared the expression of the 236 trichome genes in the 1st true leaves of the two naturally hairy *B. napus* accessions with that of the two very hairy *B. napus* transgenic lines compared to the two non-hairy accessions to identify genes that consistently had higher expression in non- hairy lines and low expression in hairy lines. This was a part of a strategy to develop lines in which expression of genes that repressed trichome development was knocked down, a strategy

that potentially could lead to a non-GMO method of developing hairy canola. Three genes were selected and RNAi knockdown constructs developed for them under the control of the leaf specific *CER6* promoter. Gene 1 is the *SPINDLY* gene, a tetratricopeptide-repeat protein with 3 copies in *B. napus* (Bo3g064270, Bo5g136400, and Bra034832), all of which are down-regulated in hairy *B. napus* leaves. SPINDLY acts as a repressor of giberrellin responses and as a positive regulator of cytokinin signalling. Arabidopsis lines carrying mutations in the *SPINDLY* gene are non-hairy. Gene 2 encodes the HDG1/HD-GL2-1/homeodomain-GLABROUS-1 protein. One *HDG* homologue Bo4g024940 was consistently down-regulated in 1st true leaves of all hairy lines tested, and had even lower expression in the 2nd leaf of *B. napus*. Gene 2 is involved in the maintenance of floral organ identity, protein acetylation and regulation of transcription, but no changes to trichomes were reported in Arabidopsis mutants. Gene 3 encodes the TRICHOME-BIREFRINGENCE-LIKE-45 (TB-like 45) protein, and 3 *B. napus* TB-like 45 homologues (Bo4g059920, Bo4g059930, Bra018347) all had very high expression in non-hairy lines compared to hairy lines. The function of Gene 3 is not clear yet, but some genes belonging to this family contribute to the synthesis and deposition of secondary wall cellulose. RNAi constructs were introduced into *B. napus* to determine if repression of these genes could increase trichome formation. The results are presented under Objective 1 above.

Leaf RNA sequencing data on all 236 trichome genes and all other expressed genes from two very hairy transgenic lines (Hairy 1 and Hairy 2) compared to their parent non-transgenic line *B. napus* cv. Westar revealed that a large number of genes (>8000 per line) were up-regulated in cotyledons of both hairy lines compared to flea beetle-susceptible Westar cotyledons, while less than 25 cotyledon genes per hairy line were down-regulated compared to Westar. The up-regulated genes include those which affect tissue toughness and involved in cell wall biosynthesis, wax biosynthesis and lignin biosynthesis. It also includes genes for metal handling, dihydroflavonols, phenylpropanoids, and glucosinolate biosynthesis and degradation. Overall stress-response genes were strongly up-regulated, including pathogenesis-related proteins and proteolysis genes which are also known to affect insect resistance. To examine this further, lignin and cellulose contents were compared in the Hairy 1 (ATGL3+), Hairy 2 (K-5-8) and Westar lines. Cellulose content was the same in all lines, while lignin content was slightly lower in the Hairy 2 line. Measurement of cotyledon and leaf glucosinolate content is still in progress.

The composition of field seed from the two transgenic hairy canola lines (AtGL3+ and K-5-8) was compared with Westar. Oil content (%) and protein content (%) were determined by near-infrared reflectance using a Foss NIRSystems Model 6500 analyzer calibrated with appropriate oilseed samples extracted with hexane. The method was based on the American Oil Chemists' Society (2005) Am 1-92 protocol

	(Determination of oil, moisture and volatile matter, and protein by near-infrared reflectance). Chlorophyll content was determined by the AOCS Official Method Ak 2-92 (AOCS, 1997) and expressed as milligrams per kilogram (mg kg^{-1}) seed. Seed oil was analyzed for fatty acid composition on an Agilent INNOWAX fused silica capillary column using an Agilent 6890N gas chromatograph equipped with a flame ionization detector (FID) (according to an American Oil Chemists' Society 2009 method) following preparation of fatty acid methyl esters (FAMEs) (Thies, 1971). FID FAME peaks were identified by comparing retention times with a FAME standard mixture GLC-428 (Nu-CHEK Prep Inc., Elysian, MN**)**. Individual fatty acids were reported as a percent of total fatty acid methyl esters by mass. Glucosinolate content was determined by capillary gas chromatography of the trimethylsilyl derivatives of extracted and purified desulphoglucosinolates (Sosulski and Dabrowski, 1984). All reps of all three lines contained 35-37% oil, 30% protein, and 13-15 μM g^{-1} total glucosinolates. The K-5-8 hairy line had higher total glucosinolate content with a mean of 20 μM g^{-1}. In particular, alkenyl-GS content was higher in K-5-8 (mean of 13%), while the AtGL3+ hairy line had 10% and Westar seed had 7%. Total methylthio-GS (0.3%) and total indolyl-GS (6%) were statistically similar for all 3 lines. Fatty acid profiles were similar for all lines, except for small increases in minor fatty acids (stearic, arachidic, and lignoceric) and a reduction in the minor acid 11-eicosenoic for the AtGL3+ line.
3. Field evaluation of flea beetle damage, and feeding preferences on trichome-modified hairy lines and on crucifer weeds.	In 2013, a flea beetle bioassay field trial was conducted from June to Oct, 2013. Three types of tests were conducted. Test (1) was designed to mimic farms where a hairy canola field might be close to a field of non-hairy canola. The most advanced tester line, Hairy Canola 2 (also known as the K-5-8 line containing the binary constructs AtGL3+::TTG1-kd RNAi) was used in this test to surround non-hairy cv. Westar. Test (2) was designed to test growth (using the K-5-8 line) and flea beetle feeding when hairy canola was planted with a flea beetle-immune Camelina biofuel crop or when hairy canola was infested with flea beetle-resistant weeds (Stinkweed and Shepherd's Purse). Test (3) was designed to evaluate the new Hairy Canola 3 (BvGL3+) line against Hairy Canola 2, a flea beetle-susceptible cv. Westar background control line, and cv. Westar treated with Helix seed treatment (to chemically protect against flea beetle feeding). Bioassays were assessed weekly for 4 weeks on cotyledons and true leaves on a 10-point rating scale detailed in Palaniswamy et al. 1992 ANOVAs (LSD and Duncan's or Tukey's statistical tests conducted to determine statistical differences between means and to rank the lines relative to one another. Naturalized lines of hairy lines of *B.napus* recovered from the diversity screening tests were not tested due to lack of greenhouse space for seed multiplication.

	The entire test was planted very late in July due to extremely wet field conditions. Flea beetle emergence and behavior was quite unusual under these conditions. Test 1. Hairy Canola 2 (AtG:3+-BnGL3-kd) plants surrounding non-hairy cultivar Westar. The only significant difference in this test was the higher growth rate for cultivar Westar grown alone compared with the reps in which Westar was surrounded by Hairy Canola 2. Flea beetle feeding was low and no statistical difference could be measured between cultivar Westar alone compared with Westar surrounded by Hairy Canola 2 for any of the reps, a result which is contradictory to normal flea beetle behavior with Hairy Canola. Test 2. Hairy Canola 2 mixed with camelina, stinkweed and shepherd's purse. Flea beetle feeding on Hairy Canola 2 alone (without inter-mixing of additional plant species) was significantly higher than plots inter-mixed with camelina or stinkweed. The shepherd's purse trial did not germinate properly. Test 3. The new Hairy Canola 3 (BvGL3+) expressing the BvGL3 trichome regulatory gene from extremely hairy *Brassica villosa*. The extremely wet spring weather after planting resulted in one rep being under water for most of this test and the remaining reps had fairly wet soil. No difference was detected in flea beetle feeding of the Hairy Canola 3 plants compared with the unprotected Westar control reps, but plant growth of Hairy Canola 3 was slightly enhanced in early seedling stages compared to Westar. Field trials in 2013 failed due to heavy rainfall and flooding of field plots in the spring. Dr. Gruber applied for a permit to conduct GMO field trials in 2014, but this was not granted due to temporary restrictions imposed by AAFC management. I was advised by AAFC management to not submit an application to conduct field trials in 2015 due to the illness of the principal investigator (M. Gruber) and retirement of field insect agronomist J. Soroka. Priority was directed to the development of the additional tester lines and this milestone was removed in the amended contract.
4. Evaluation of drought tolerance on optimum hairy lines.	Two laboratory-based drought tolerance tests have been designed. a) Water release (transpiration) rates from hairy leaves and stems under low watering conditions using leaf clip-on transpiration sensor boxes. Hairy and non-hairy plants were grown under equivalent conditions in a specialized growth chamber and watered equivalently. b) Growth with watering, followed by a drought period, followed by re-watering, with measurements of days-to-wilting, days-to-recovery from wilting, number of yellowed leaves, biomass yield, and seed

	yield/weight. Young trichome-bearing leaves on the K-5-8 line and non-hairy leaves of the Westar parent plant were evaluated. Young plants were grown under usual watering and growth conditions in a greenhouse and analyzed at 2.5 weeks and 4 weeks using a clip-on CiSD photosynthetic gas exchange meter. The meter measured leaf surface radiation, leaf/chamber temperature, flow rate, atmospheric pressure, substomatal CO_2, transpiration rate, stomatal conductance of water, and photosynthetic rate. Initially, plants were grown and tested under controlled greenhouse conditions, but with changing fan operations. In a 2nd experiment, plants were grown in the greenhouse, but transferred prior to testing for a 24 hour acclimation period into a controlled environment chamber, which had no other plants and a consistent fan operation. Six pots with 1 plant per pot were grown, and each of the flattest and largest 1st t-to-4th true leaves on at least 4 plants per line were tested using a 1-min time-span per test, with 3 replicate tests per leaf. Plants were removed from their growth position within the growth cabinet one-by-one and placed onto the same position when testing to improve measurement consistency. Atomospheric pressure, temperature, and flow measured by the clip-on CiSD photosynthetic gas exchange meter were highly consistent between all plants per line and across the two lines in both experiments. For other measurements, technical replicates per leaf were consistent, but re-measurement of each plant in a different order after a 1 hour recovery time differed from the initial measurements. To improve consistency, a new design was adopted for use in the next reporting period where 15-20 plants per line were grown in a randomized plot design, then measurements taken on the 1st leaf at 2.5 weeks, followed by repeat measurements after a 24 h recovery rate, followed by another growth period and two similar measurements of the 4th leaf. All measurements were conducted on plants sampled using the same plant assessment order for the entire set of plants each time. Atomospheric pressure, temperature, and flow as measured by the a CiSD photosynthetic gas exchange meter were similar on young trichome-bearing leaves on the K-5-8 line and non-hairy leaves of the Westar parent plant.
5. Release of 1st offering of hairy canola germplasm to plant breeding community.	In addition to the publications noted below, the results of the work to evaluate trichome density in nearly 1,000 Brassica accessions was presented at the Canola Industry meeting in 2012. This was followed by a meeting with JoAnne Buth (Head of the Canola Council of Canada) at the AAFC-SRC and concluded with a promise to promote/publicize the results of this germplasm study. After Ms. Buth left the organization to become a senator, communication with the Canola Council continued through Denise Maurice (VP Crop Production) until his untimely death. The AAFC Business Office was subsequently charged with sending notifications to plant breeding companies. In addition, Dr. Gruber actively promoted the germplasm resource and the technology leading to the transgenic hairy canola through her network of contacts within the major canola breeding

	companies.

add additional lines as required

8. Discussion: *Provide discussion necessary to the full understanding of the results. Where applicable, results should be discussed in the context of existing knowledge and relevant literature. Detail any major concerns or project setbacks.*

A thorough discussion of the results is available in the publications appended to this report.

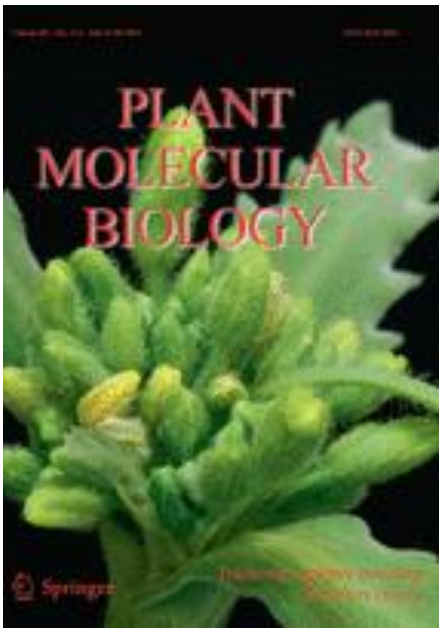
9. Conclusions and Recommendations: *Highlight significant conclusions based on the previous sections, with emphasis on the project objectives specified above. Provide recommendations for the application and adoption of the project.*

I was only involved with the project for the final 1.5 - 2 years to supervise the activities of the last post-doctoral fellow, but the project appears to have been highly productive and successful. I have elected to combine sections 9 (conclusions) and 10 (success stories) here as they tend to address the same elements with respect to this particular project.

Conclusions Stories:

1. Natural variation for trichome production in Brassica species.

One of the more ambitious aspects of the project was to phenotype nearly 1000 Brassica accessions for trichome abundance and physical features. This work identified natural accessions of *B. napus* and *B. rapa* with moderate levels of trichomes and a *B. villosa* line with extremely high levels of trichomes on all parts of the plant. This information will serve as a tremendous resource for breeders aiming to introduce insect, drought and freezing tolerance into canola through the trichome production. A description of the *B. villosa* system for studying non-glandular trichomes and genes in the Brassicas was published in the journal Plant Molecular Biology (see Nayidu et al., 2014 below) and was featured on the cover.



2. Naturalized hairy *B. napus* lines

The phenotyping work above revealed two natural *B. napus* lines with moderate levels of trichomes. Crossing of the lines yielded progeny with higher levels of trichomes than the parents. The trait was fixed by selfing for four generations and generating doubled haploid lines from high trichome lines. This material will likely be of most interest to breeders and in the future should be tested in the greenhouse and field for flea beetle resistance and other agronomic characteristics. The retirements of Drs. M. Gruber, J. Soroka and B. Elliott have limited AAFC's ability to conduct insect field trials and related agronomy research, but I will attempt to secure the resources and expertise needed to further this research.

3. Hairy Canola 1 and Hairy Canola 2.

The project was based on earlier work which demonstrated that expression of the Arabidopsis *GL3* gene in *B. napus* greatly increased trichome production. This line was denoted Hairy Canola 1, but it suffered from some growth abnormalities. These problems could be corrected by reducing the expression of another gene, *TTG1*, through RNAi which yielded a line with high levels of trichomes and normal growth. This line was denoted Hairy Canola 2. Laboratory and field assays showed that these lines deterred flea beetle feeding and exhibited greatly reduced damage even under high flea beetle pressure. My discussions with Dr. Gruber after her retirement indicated that uptake of this material by the private sector was less than anticipated for several reasons: 1) Restrictions exist on the use of the *AtGL3* gene through a patent issued to Texas A&M University; 2) Most of the major canola breeding companies are also chemical companies, some of whom manufacture chemicals currently used for flea beetle control. She was informed that they would not likely be interested in developing technology if it compromised another aspect of their business; 3) The phenotype requires the over-expression of one transgene (*AtGL3*) and another transgene to reduce the expression of an endogenous gene (*TTG1*) which may be too complicated to enter into already complicated private-sector breeding programs.

4. A better understanding of why canola does not produce trichomes

This work above, however, clearly demonstrated that trichome abundance is linked to flea beetle resistance and that the *GL3* gene in contemporary canola varieties is likely non-functional. Many such traits appear to have been lost in the genetic bottleneck that arose over that past few decades as canola breeders focused on seed quality and productivity at the expense of agronomics. The project attempted to identify functional *GL3* orthologs in other Brassica species that would not be encumbered by existing intellectual property. This work was partially successful in that *GL3* genes were identified in hairy *B. rapa* lines that lead to the production of trichomes in *B. napus*, though not to the same extent as *AtGL3*. Comparison of GL3 protein sequences and testing of AtGL3-Brassica GL3 chimeric GL3 proteins also indicated that the *B. napus* GL3 in current varieties may have mutations that render it non-functional. Attempts were also made to understand why the GL3 is not working in *B. napus* and this appears to due to a poorly functioning BnGL3-1 protein and the expression of a non-functional BnGl3-2 homeologue that interferes with the function of GL3-1. Gene expression studies comparing the hairy *B. villosa* line to non-hairy *B. napus* lines revealed that this may also be due, in part, to differences in the expression of other members of the GL3 pathway.

10. Success stories/ practical implications for producers or industry: *Identify new innovations and /or technologies developed through this project; and elaborate on how they might impact the producers /industry.*

See section 9 above.

11. Patents/ IP generated/ commercialized products: *List any products developed from this research.*

No new IP was generated. Please seed Objective 5 for an explanation as to how the information from the projects was made available.

12. List technology transfer activities: *Include presentations to conferences, producer groups or articles published in science journals or other magazines.*

Publication in peer-reviewed scientific journals

Taheri, A., Robinson, S.J., Parkin, I.A.P. and Gruber, M.Y. (2012) Revised selection criteria for candidate restriction enzymes in genome walking. PLoS ONE 7(4): e35117. (doi:10.1371/journal.pone.0035117)

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Alahakoon, U.I., Adamson, J.B., Grenkow, L.A., Soroka, J.J., Bonham-Smith, P.C. and Gruber, M.Y. (2016). Field growth traits and insect-host plant interactions of two transgenic canola (Brassicaceae) lines with elevated trichome numbers. Can. Entomol. 00:1-13. epub ahead of print (doi:10.4039/tce.2016.90

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Gruber M, Alahakoon U, Taheri A, Nayidu N, Zhou R, Aung, B., Sharpe. A., Hannoufa, A., Bonham-Smith P, and Hegedus D. The glabrous cotyledon transcriptome of transgenic hairy-leaf and ultrahairy-leaf *Brassica napus* lines.

Alahakoon, U., Zhou, R., Bonham-Smith P, and Hegedus D. Seed composition of hairy-leaf and

ultrahairy-leaf *Brassica napus* lines.

Gruber, M., Naidu, N., Taheri, A., Gruber, M., Parkin, I., Sharpe, A., and Hegedus, D. A comparison of trichome gene expression in naturally hairy and non-hairy accessions of *Brassica napus*, *B. rapa*, and *B. oleracea*.

Heydarian Z, Yu M, Gruber M and Hegedus D. The impact of replacing *Brassica napus* GL3 subdomains with those from *Arabidopsis thaliana* GL3 on trichome production, growth and development.

Conferences and non-reviewed papers:

Taheri, A., Nayidu, N.K., Gao, P., Yu, M., Regan, S., Parkin, I., and Gruber, M.Y. (2012.) Battling flea beetle with genomic approaches that dissect trichome (hair) development and diversity. Poster. Canola Industry Meeting and 9th Applying Genomics to Canola Improvement Workshop, Saskatoon, SK. Dec 5-6.

Taheri, A., Alahakoon, U., Nayidu, N.K., Sharpe, A., Parkin, I., and Gruber, M.Y. (2013) Battling insects with genomic approaches that dissect trichome (hair) development and diversity. Cold Spring Harbour Symposium.

Media Interviews and Stories on Hairy Canola Research:

1. Arnason, R. 2013. Do regulatory issues hurt farmers? Western Producer Nov. 28.

2. Guenther, L. 2013. Battling flea beetles with hairy canola. Front Page selection. Grainews. Oct. 21. *http://issuu.com/fbcpublishing/docs/131001154109014e7fcd193c4e3e96fb026bea633245?e=0*

3. Lockrey-Wessel, D. 2013. "Hairy" Canola brushes off flea beetles. Innovation Express (Agriculture and Agri-Food Canada) 4: pg. 6.

13. List any industry contributions or support received.

The project was co-funded by SaskCanola and a copy of this final report will be submitted to them.

14. Is there a need to conduct follow up research? *Detail any further research, development and/or communication needs arising from this project.*

The early part of the project was devoted to resource development with many of the most exciting studies being initiated near the end of the project. Should resources ever become available, I foresee the following as being worthy pursuits:

1. *Natural hairy B. napus lines.* The naturalized hairy *B. napus* lines derived from a cross between moderately hairy lines should be moved forward to greenhouse and field evaluation for flea beetle resistance and other agronomic properties. This will require the involvement of AAFC and private sector entomologists, agronomists and breeders.

2. *Exploitation of B. villosa as a resource for insect, drought and heat tolerance traits in B. napus. Brassica villosa* is closely related to *Brassica oleraceae*, possibly a sister-species. It carries the Brassica C genome that is shared with *B. napus* and it is possible that the extremely hairy trichome trait could be accessed through wide crosses or re-synthesis.

Though I have no personal experience with this, consultations with AAFC researchers indicate that this is possible.

3. *Repair of the B. napus GL3*. There is reasonably good evidence that the loss of the ability to form trichomes in current *B. napus* varieties is due to the presence of a non-functional *GL3* allele. We know that the Arabidopsis GL3 alone can induce adundant trichome formation in *B. napus* and we made an attempt to understand what part of the *B. napus* GL3 protein is non-functional by generating chimeric Arabidopsis-*B. napus* GL3 proteins. While these were capable of inducing trichome formation, they did not work as well as the AtGL3. Comparison of the two proteins shows that only a few major differences exist. Testing of a few more constructs would likely pinpoint the mutation(s) that most impact the function of the *B. napus* GL3. These could then be repaired using advanced breeding tools, such as CRISPR gene editing; we have experience working with this technology in other oilseed crops.

15. Acknowledgements. *Include actions taken to acknowledge support by the Ministry of Agriculture and the Canada-Saskatchewan Growing Forward 2 bilateral agreement.*

The following statement was included in the Acknowledgements section of any publications arising from the project:

"This research was supported by grants from the Canola Council of Canada, the Western Canadian Canola Grower organizations, the Agriculture Development Fund of the Government of Saskatchewan, the Saskatchewan Canola Development Commission, and funding from Agriculture and Agri-Food Canada."

16. Appendices: *Include any additional materials supporting the previous sections, e.g. detailed data tables, maps, graphs, specifications, literature cited*

1. Figures noted in report
2. Financial statement
3. Project publications