AAFC RESEARCH BRANCH
Research Project Final Report
Developing Innovative Agri-Products Program (Vote 10 Funding)

Project Title:	Activity B.2.4. Identify the molecular basis of green seed in canola and develop gene-based markers for degreening
Start Date (yyyy-mm-dd):	2011 Jan 1st
Expected End Date (yyyy-mm-dd):	2013 March 31st
Actual End Date (yyyy-mm-dd):	2013 April 30th
Principal Investigator (PI):	K. Peter Pauls

Short Executive Summary of report:

The presence of green seed in canola at harvest is unwanted, because chlorophyll in the oil is undesirable from a consumer's perspective and leads to rancidity in the oil. Grade #1 canola contains a maximum of 2.0% green seed. Green seeds occur especially when an early stress event destroys the enzymatic activity that normally breaks down the chlorophyll into colourless compounds.

The current work was based on the hypothesis that genetic variation for the susceptibility of canola lines to stay green exists and sought to find molecular markers related to the trait.

The work on the current project was focused on:

- defining a laboratory treatment of canola that induces the green seed phenomenon
- identifying canola germplasm that differed in the green seed incidence after a cold treatment during the seed maturation period, and
- identifying gene targets that might be involved in the stay-green phenotype in canola.

The work demonstrated that the severity of low temperature damage to canola seed and the resulting occurrence of green seed in the crop depend on the maturity of the seed during the cold period, the severity of the cold treatment and the variety (genotype).

It defined a laboratory treatment for inducing the green seed phenomenon (four to 50 fold) in a consistent manner that included: growing plants from various varieties in a growth room (at 22°C light/16°C dark); hand pollination after bolting and prior to flowering; controlled freezing in a programmable freezer twenty days after pollination (DAP) to -4°C; recovery and regrowth of the plants in the growth room until maturity and assessment of the percentage of green seed.

It identified a set of six canola varieties that differ in their susceptibility to form green seed during a cold stress and showed that the differences observed among the varieties in the laboratory screen were correlated with the frequencies of green seed observed in the OOPSC Spring Canola Co-op Trials (2009).

The work also examined global gene expression patterns in control and treated seed by RNA-Seq to examine the expression patterns of genes coding for enzymes in the degreening process in normal and freeze-treated tissues. The differences between treated and control and sensitive and tolerant genotypes are being used to identify correlations between gene expression and the stay green trait.

In the future, polymorphisms in the genes associated with the stay green trait can be used for selecting for lines that have a low incidence of stay green.

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A. Research Progress and Accomplishments (to date in relation to expected milestones and deliverables / outputs)

- Include brief summary of:
  - Introduction, literature review, objectives, milestones and deliverables / outputs.
  - Approach / methodology (summary by objectives).
- Include results and discussion (overview by objectives and milestones), next steps and references.

Introduction / literature review

Green seed in canola at harvest is unwanted, because chlorophyll in the oil is undesirable from a consumer's perspective and leads to rancidity in the oil. Under typical field growth conditions, canola (*Brassica napus*) seeds contain chloroplasts during early seed development and then catabolize the photosynthetic machinery during seed maturation, producing mature seeds at harvest that are essentially free of chlorophyll (Chl). However, frost at a critical time after flowering, can result in significant grade loss (Johnson-Flanagan and Thiagarajah, 1990). Grade #1 canola contains a maximum of 2.0% green seed, and frost resulting in green seed is the major cause of downgrading in canola. Green seed occurs due to a failure of the seed to complete the normal chemical processes involved in degreening.

Genetic variation for the susceptibility of canola lines to stay green is thought to exist. Several enzymatic steps in the degreening process that occurs during senescence have been examined and identified in a variety of plants, including canola, tobacco and Arabidopsis. From a study comparing degreening in frozen and non-frozen developing canola seeds, it was suggested that freezing inhibits seed degreening by interfering with the induction of 'Pheophorbide a Oxygenase' (PaO) activity (Chung et al 2006). A chloroplast-located and senescence-induced hydrolase, that specifically dephytylates the Mg-free chlorophyll (called pheophytinase; PPH), has been shown to be a critical enzyme in chlorophyll breakdown in Arabidopsis. Mutants for this gene exhibit a stay green phenotype (Schelbert et al 2009).

In spite of the importance of the green seed problem in canola, no method for evaluating the susceptibility/resistance of Canadian varieties to frost and the formation of green seed has been described and, other than the information available in the OOPSC Spring Canola Co-op Trials (2009) reports, no systematic evaluation of Canadian canola varieties (performed under controlled conditions) for the problem exists.

The overall goal of the current work was to enhance the understanding of the problem and develop methods to allow canola breeders to screen for germplasm with reduced incidence of green seed. The use of tools such as molecular markers can accelerate selection of plants with certain traits; this allows plant breeders to rapidly screen large populations of plants for those that possess the trait of interest. The screening is based on the presence or absence of beneficial forms of certain genes (alleles) as determined by simple laboratory procedures, rather than by phenotypic appearance (field).

More recently, direct sequencing of transcripts by high-throughput sequencing technologies (RNA-Seq) has become an additional alternative to microarrays and is superseding serial analysis of gene expression (SAGE) and massively parallel signature sequencing (MPSS) (Yamada et al., 2003; Bertone et al., 2004; David et al., 2006). Like SAGE and MPSS, RNA-Seq does not depend on genome annotation for prior probe selection and avoids biases introduced during hybridization of microarrays.

RNA-Seq can be used for gene identification, polymorphism detection and transcript profiling in canola seed. Using RNA-Seq has several advantages over other technologies: unlike hybridization-based technologies such as microarrays, RNA-Seq does not require pre-existing sequence information, and candidate genes identified in this study will be a valuable resource for advancing genetic/genomic research in canola and eventually for improving the quality of canola seed.

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Objectives/ milestones

To address the lack of information about the occurrence and susceptibility of canola to frost induced green seed the current project had the following objectives, to:

- define a laboratory treatment of canola that induces the green seed phenomenon,
- identify canola germplasm that differs in the green seed incidence after a cold treatment during the seed maturation period, and
- identify gene targets that might be involved in the stay green phenotype in canola and could be used to develop markers of selection of lines with low green seed incidence.

Deliverables/ outputs

The anticipated outputs of this project were:

- a description of a method to assess the susceptibility of canola germplasm to frost-induced green seed;
- confirmation of genotype-based difference in the susceptibility of canola varieties to cold-induced green seed, and
- the identification of gene expression differences between canola varieties that degreen or stay green that can be used as selection criteria for varieties that have a reduced incidence of green seed.

Approach/ methodology

Objective A. To define a laboratory treatment of canola that induces the green seed phenomenon.

Initial studies were conducted with spring canola varieties Westar, Topaz and InVigor5030. The plants were grown in a growth room on a 12-h photoperiod at 22°C light/16°C dark and a relative humidity of 70%. The plants were fertilized weekly. After bolting and prior to flowering, inflorescences with similar maturity were chosen from each canola plant for hand pollination. The tip of each flowering bud was cut open and hand pollinated. Each pollinated bud was tagged with the pollination date. At different days after pollination the plants were subjected to different freezing temperatures (-3ºC, -5ºC and -7ºC) by moving them outdoors (in Jan) for a few hours (3-10h) during the dark period and then returning them to the growth room. After maturity, water was withheld from the plants for two weeks and the percentage of green seed was determined.

Objective B. To identify canola germplasm that differs in the green seed incidence after a cold treatment during the seed maturation period.

Records of the OOPSC Spring Canola Co-op Trials (2009) for several years were examined for incidence of green seed. On the basis of the values over several years, contrasting lines were selected for additional testing with the freezing procedure; including low green seed lines [5020 (0.8%), 7145RR (0.7%), 8440 (0.6%)] and high green seed lines [6020RR 4.3%), OAC C09-01 (2.6%), OAC 09-02 (1.7); OOPSC Spring Canola Co-op Trials (2009) [Single Year Quality/Agronomic Traits Summary].

Plants from the six lines were grown as described above and hand pollinated as above. Six sets of plants were freeze-treated 20 days after pollination (DAP) in a darkened, controlled-environment freezer beginning at 22ºC with high humidity, and cooled at 5ºC/h until the temperature reached -4ºC. This temperature was held for six hours and then increased 5ºC/h until it reached 22ºC. The freeze-treated plants were returned to the growth chamber where the control plants were kept and seeds were collected at maturity and assayed for the green seed incidence.

Objective C. To identify gene targets that might be involved in the stay-green phenotype in canola and could be used to develop markers of selection of lines with low green seed incidence.

The initial approach for identifying gene targets was to analyze for transcripts from the 'Pheophorbide a Oxygenase' (PaO) gene, as this was reported to be a key control point in the overall regulation of chlorophyll degradation and that freezing interfered with the induction of PaO activity that typically occurs

in the later phases of canola seed development (Chung et al 2006).

In the second year, the molecular approach shifted to a direct comparison of the transcriptomes of the seeds that developed after the freezing treatment with those that derived from untreated plants - using the RNAseq procedure. Six lines with differing green seed incidence (5020, 7145, 6020RR, OAC C09-01, OAC C09-02 and 844) were used. Seed was collected from 36 plants (three replications per line control; three replications per line freeze treated) treated with the freezing protocol (20 DAP), as described above. The seeds were collected 36 DAP and were preserved quickly in liquid nitrogen and stored at -80ºC freezer for RNA isolation.

A modified rapid and effective method was used for the isolation of RNA from immature canola seed, based on homogenization in a simple CTAB (hexadecyl trimethyl-ammonium bromide) buffer and purification on a silica column (Qiagen RNA mini extraction kit). Absorbance ratios (260/280 nm) were measured for all of the samples to determine purity, and the intactness of the RNA was determined by examining the rRNA bands after gel electrophoretical separation with 1.4% of agarose.

Illumina TruSeq RNA Sample Prep Kit v2 was used to prepare the cDNA libraries for RNA-Seq. The 36 cDNA libraries are being sequenced by The Centre for Applied Genomics (TCAG) at The Hospital for Sick Children in Toronto (www.tcag.ca). The TCAG conducts a digital expression profile analysis of transcripts in each sample as well.

Results/ discussion

Objective A. To define a laboratory treatment of canola that induces the green seed phenomenon.

The preliminary experiments indicated that the freezing treatments effectively induced the green seed phenotype. In the harshest treatment (-7°C for 10 h, 37 days after flowering) 38-65% of the seeds were green across the three varieties. In the milder treatments (-3°C for 10h, 22 days after flowering) the green seed incidence was 1%, 2% and 6% in Topas, Invigor5030 and Westar, respectively. Therefore, the experiments established that the susceptibility to green seed formation after a frost treatment was genotype dependent. In all the treatments the highest incidence of green seed was found in the Westar samples. The greatest differences between treatments were observed at the more moderate treatments (-4 °C) given earlier in the seed maturation period.

On the basis of these experiments, a protocol for assessing the susceptibility of canola consisted of the following steps:

1. Canola plants were established in separate pots in a growth room on a 12-h photoperiod (22°C light/16°C dark) and fertilized weekly.
2. After bolting and prior to flowering, plants with inflorescences at the same maturity were chosen and they were hand pollinated and tagged with the pollination date.
3. 20 days after pollination the plants were subjected to a freezing treatment in a darkened, controlled-environment chamber. The chamber was initially set at 22°C with high humidity, the temperature was decreased at 5°C per hour until it reached -4°C, and held at that temperature for six hours. The temperature was then increased at 5°C per hour until it reached the initial temperature of 22°C.
4. The plants were returned to the growth room and sampled for RNA or kept until maturity and analyzed for green seed content.
5. Some of the plants were maintained in the growth room as controls and were, therefore, not subjected to the freezing treatment.

Objective B. To identify canola germplasm that differs in the green seed incidence after a cold treatment during the seed maturation period.

The treatment of the six canola lines (5020, 7145RR, 8440, 6020RR, OAC C09-01, OAC 09-02) with the

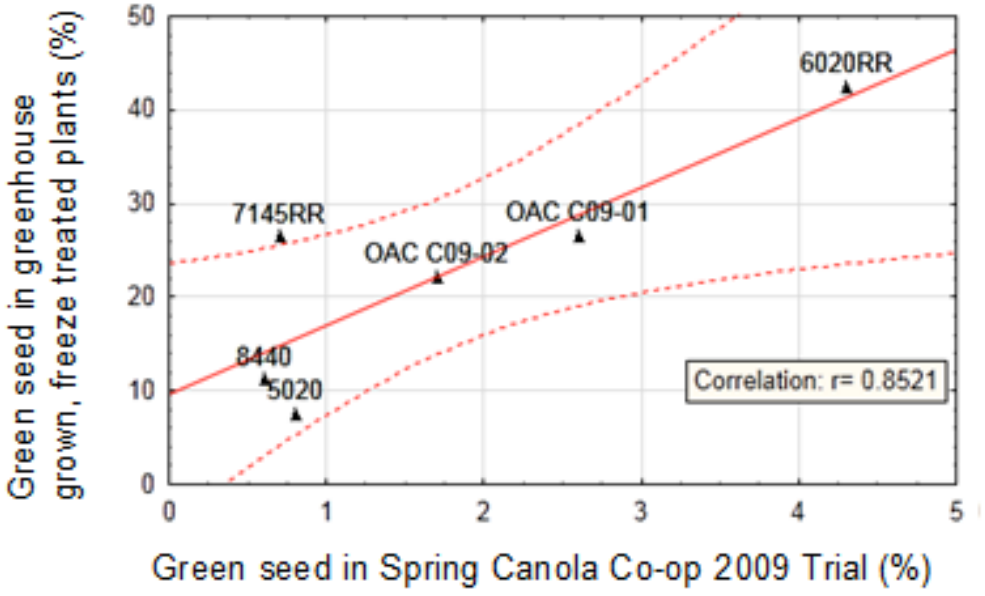
freezing procedure described above resulted in large increases (four to 50 fold) in the incidence of green seed in the treated versus control plants. Large differences in the incidence of green seed occurred among the treated plants [high (42%) 6020RR; medium (22-26%) 7145RR, OAC C09-01, OAC 09-02; low (8-11%) 5020, 8440)] (table 1). These results confirm that genetic variability exists for this trait.

Table 1. Incidence of green seed in canola lines treated with at -4°C for six hours compared to control (non-treated) samples and occurrence in OOPSC Spring Canola Co-op Trials (2009) Single Year Quality/ Agronomic Traits Summary.

Entry	Days to Flowering	Green Seed (%)		
		Green House		Spring Canola Co-op 2009 Trial
		Control	Treated	
5020 (Bayer)	51	2.0	07.6	0.8
7145RR (Monsanto)	51	0.5	26.5	0.7
6020RR (Brett Young Seeds)	51	6.2	42.5	4.3
OAC C09-01 (UofG)	55	2.2	26.6	2.6
OAC C09-02 (UofG)	54	1.2	22.2	1.7
8440 (Bayer)	51	0.8	11.2	0.6

The results of the freezing tests on the different canola varieties were correlated with the incidence of green seed in the OOPSC Spring Canola Co-op Trials (2009; figure 1), suggesting that the growth room screen will have real utility in identifying lines with high and low incidence of green seed in the field.

Figure 1. Correlation between incidence of green seed in lab freezing test and in OOPSC Spring Canola Co-op Trials (2009).



<u>Objective C.</u> To identify gene targets that might be involved in the stay-green phenotype in canola and could be used to develop markers of selection of lines with low green seed incidence.

The total RNA isolated from the 36 seed samples (six varieties; three replications per variety of controls; three replications per variety of freeze treated samples) was of good quality and had good yields as determined by photometric and electrophoretic analyses. All of the samples had concentrations greater than 1ug/ul and their 260/280 absorbance ratios were >2. Also, the electrophoretic patterns showed that the total RNA was intact, since the patterns had distinct 18S and 28SrRNA bands and the 28S band was roughly twice as bright as the 18S band. These results indicated that the RNA is of the appropriate quality needed to construct the cDNA libraries for RNA-Seq.

Presently, the cDNA libraries construction is in process. The 36 cDNA libraries are being sequenced by The Centre for Applied Genomics (TCAG) and the Centre is also preparing a digital expression profile of the transcriptome in each sample that identifies the genes from which the transcripts are derived and their abundance.

<u>Next steps</u>

From comparisons of the differences in the transcriptomes between varieties that are susceptible to the freezing-induced green seed [high incidence (42%) 6020RR; medium (22-26%) 7145RR, OAC C09-01, OAC 09-02] with those that are not [low incidence (8-11%) 5020, 8440)] correlations between transcripts (involved in chlorophyll degradation) will be identified that are differentially sensitive to freezing between the lines.

These gene-based differences will be good marker candidates for selecting canola lines with low green seed incidence.

References

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B (I). Funded Collaborators (Co-PI, AAFC, other federal scientists)

- Include the name of scientist / organization.

B (II). Acknowledgement of non-funded collaborators (who provide support, e.g. access to other laboratory or other facilities and equipment input / advice / guidance / assistance, etc).

- For research supported by targeted funding programs (e.g. DIAP, Clusters, etc.) please list any collaborators who are receiving Contribution Vote 10 funds (e.g., university and industry collaborators). In addition, please list separately the participants who support your project but are not receiving any funding through the program.
- Include name of scientist / organization.

Sergio Pereira, The Centre for Applied Genomics (TCAG), The Hospital for Sick Children, Toronto

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C. Variance Report (if applicable, describe how the work differs from the proposed research)

- Include changes to objectives and project work plan / budget, changes to the team, other constraints.

Initially it was proposed to examine the expression patterns of several of candidate genes coding for enzymes in the degreening process in untreated and freeze treated tissues to identify correlations between gene expression and the stay green trait. The initial approach to identifying gene targets to analyze was to focus on the 'Pheophorbide a Oxygenase' (PaO) gene as this was reported to be a key control point in the overall regulation of chlorophyll degradation and that freezing interfered with the induction of PaO activity that usually occurs in the later phases of canola seed development. Genomic DNA from young leaves of six canola varieties (Dynamite, Dorothy, Colossus, Q2, Westar, Avalanche and Topaz) was extracted and used for PCRs with primers designed for *Brassica napus* BnPaO2 cDNA nucleotide sequence to determine, if there are useful polymorphisms in this gene that might be used to develop markers. The initial results from this approach were not promising.

Therefore, the molecular approach was shifted to a direct comparison of the transcriptomes of the seeds that develop after the freezing treatment with those that have not been treated using the RNAseq procedure. The advantages of this approach were that the analysis gives information about all the genes that are expressed in these tissues, it will allow us to distinguish transcripts from different copies of the genes and it provides quantitative information about transcript abundance.

However, the procedures to isolate good quality RNA in appropriate quantity for the RNAseq experiments from seed tissue took longer to develop than anticipated and the last step still needs to be completed, namely the analysis of the transcripts from the various samples.

D. Impact Assessment (if applicable, describe how the variance factors above will impact project continuation)

- Include changes to the objectives, changes to the project work plan / budget, changes to performance (i.e. meeting targets).

The established sequence and molecular marker resources will improve and promote genetic and genomic research as well as genome-based breeding in canola. The identification of gene expression differences between canola varieties that degreen or stay green can be used by canola breeders as selection criteria to select lines that degreen properly in short season environments in Eastern Canada. Reduced green seed in canola crops will reduce the susceptibility of the oil to oxidize and become rancid. Therefore, candidate genes identified in this study will be a valuable resource for advancing genetic/genomic research in canola and eventually for improving the quality of canola seed.

E. Achievements (include only those related to this project)

- Include innovations, publications / conferences, technology transfer, capacity building, success stories, media, recognition and other outputs.

The work demonstrated that the severity of low temperature damage to canola seed and the resulting occurrence of green seed in the crop depend on the maturity of the seed during the cold period, the severity of the cold treatment and the variety (genotype).

It defined a laboratory treatment for inducing the green seed phenomenon in a consistent manner (four to 50 fold) that included: growing plants from various varieties in a growth room (at 22°C light/16°C dark); hand pollination after bolting and prior to flowering; controlled freezing in a programmable freezer twenty days after pollination to -4 °C for six hours; recovery and regrowth of the plants in the growth room until maturity and assessment of the percentage of green seed.

It identified a set of six canola varieties that differ in their susceptibility to forming green seed during a cold stress and showed that the differences observed among the varieties in the laboratory screen were correlated with the frequencies of green seed observed in the OOPSC Spring Canola Co-op Trials (2009). In particular, it categorized the incidence of frost-induced green seed in the varieties as: high (42%) 6020RR; medium (22-26%) 7145RR, OAC C09-01, OAC 09-02; or low (8-11%) 5020, 8440). Thus, it confirmed that genotype-based differences exist in the susceptibility of canola varieties to frost-induced green seed incidence.

The results of the freezing tests on the different canola varieties were correlated with the incidence of green seed in the OOPSC Spring Canola Co-op Trials (2009), suggesting that the growth room screen will have real utility in identifying lines with high and low incidence of green seed in the field.

The work also modified a rapid and effective method for the isolating RNA from immature canola seeds that is based on homogenization in a simple CTAB (hexadecyl trimethyl-ammonium bromide) buffer and purification on a silica column (Qiagen RNA mini extraction kit) that should be applicable to a wide range of gene expression studies in developing canola seeds.

The work also examined global gene expression patterns by RNA-Seq to identify differences between treated and control plants as well as sensitive and tolerant genotypes that could be used to develop gene-based markers for the low susceptibility to green seed in canola. In the future, such markers could be used to select lines that have a low incidence of stay green.

F. Lessons learned (self-evaluation of project)

Overall, the project made significant advances in establishing evidence for variety-based (genetic) variation in the susceptibility of canola to frost-induced green seed incidence. This suggests that there is a good possibility of selecting for low incidence of green seed and higher quality stability, if good selection protocols are available.

However, no systematic screen for the green seed trait existed and good progress was made in developing a laboratory method for evaluating canola lines for their susceptibility to frost-induced green seed, based on exposure of hand-pollinated plants 20 DAP to -4 °C for six hours and assessment of green seed incidence at maturity. The high correlation of the values obtained by the laboratory method and the values measured in the OOPSC Spring Canola Co-op Trials (2009) suggest that the screen will be useful for evaluating breeding material.

Also, excellent progress was made in the molecular analysis of differences in gene expression between resistant and susceptible lines. However, some difficulties were encountered in isolating good quality RNA from seeds caused by high levels of polysaccharides, polyphenols, and lipids that degraded or co-precipitated with the RNA. The time required to trouble shoot the RNA isolation methodology delayed some of the work on the identification of molecular markers that might be used in the future to select plants resistant to the frost-induced green seed condition. Nevertheless, the RNA isolation protocol that was developed should have application in a wide range of gene expression studies in developing canola seeds that may be related to a wide range of quality traits.

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K. Peter Pauls		K. Peter Pauls Digitally signed by K. Peter Pauls Date: 2013.05.29 12:02:35 -04'00'
PI Name	**Date**	**Signature**

Note: After completion and signature, this report must be provided to the appropriate Science Director for assessment. A PDF copy of this report will be sent to Science Operations by the Science Director's office along with the project assessment.