Government of Saskatchewan
Ministry of Agriculture

1. Project title and ADF file number.

File number: 20100098

Genomics of Clubroot disease development in canola and development of *in planta* RNAi to impart novel resistance.

2. Name of the Principal Investigator and contact information.

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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.*

In the absence of a pathogen genome, and in order to identify new resistance possibilities in canola to clubroot disease, a cDNA library from *Plasmodiophora brassicae*-infected canola tissues at 35 days post inoculation was constructed. From this library 15 putative effector sequences (pathogen secreted proteins that aid pathogen infectivity) were identified as not being expressed in resting spores but expressed at later stages of infection and disease progression. Of these, 10 are associated with plant Programmed Cell Death (a plant process to inhibit pathogen spread) and are being studied further as possible tools for improved plant resistance. The subsequent publication of the *P. brassicae* genome (2015) provided the project with a reference genome against which we are able to map our infected versus non-infected transcriptome data. While still being analysed, these data are providing the necessary insights into the plant-pathogen interaction network(s) that highlight the pathogen's successful ability to escape detection, throughout its complete intracellular life cycle, by the plant. Once identified, we will be able to target key stages in these networks to effectively unmask the pathogen and thereby present it to the plant's 'immune' system for degradation. To guide us in this work we have established a tool box of staining technologies that allow us to track the intracellular life-cycle progression of *P. brassicae*. This project has successfully positioned our clubroot research group for future success in the utilisation of pathogen protein targets for improved resistance to clubroot disease in canola.

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

Clubroot, resulting from an infection by *Plasmodiophora brassicae* Woronin, is a devastating disease of Brassica, especially canola (*Brassica napus*). Clubroot disease arises from a compatible interaction between the susceptible host plant and virulent *P. brassicae*. However, a plant resistance (*R*) gene, encoding a plant immune receptor could recognize the pathogen-derived avirulence *Avr* gene product resulting in a highly efficient plant defence reaction being activated. Because *R* gene-based resistance is based on the recognition of a single pathogen-derived effector, the resulting resistance is highly specific and usually easily overcome by successive generations of the pathogen. Clubroot resistant varieties of canola have been identified, however, where this resistance relies on a single *R* gene we are seeing erosion of the level of resistance due to pathogen evolution (Canola Council of Canada 2014). One way to establish new resistance in canola is to identify 'new' *R* genes in resistant relatives and to incorporate these genes into the canola germplasm. In this way, four *R* genes have recently been identified; *Rpb1* & *Rpb3* on chromosome A3 (*B. rapa*), *Rpb2* on chromosome B5 (*B. nigra*) and *Rpb4* on chromosome A8 (*B. rapa*) (Chu *et al* 2015).

Previous to our studies, only a few effector proteins had been identified from *P. brassicae*; i) PbPPI1 - shares identity with several proteins found in other intracellular pathogens (Engleberg *et al* 1989; Moro *et al* 1995; Bulman *et al* 2006; Siemens *et al* 2009), ii) PbPDA1 - containing a 'NodB-type' chitooligo-saccharide deacetylase domain known to deacetylate substrates including xylan and chitin and as such may play a role in cell wall penetration and early colonisation, and iii) PRO1 - a serine protease suggested to be involved in clubroot pathogenesis by stimulating resting spore germination through proteolytic activity (Feng *et al* 2010). The lack of knowledge of the molecular biology of *P. brassicae* infection, clubroot disease development and the mode of action of secreted effector proteins and *R* genes,

has seriously hampered the development of molecular tools and strategies for imparting long term durable resistance into canola breeding programs via resistance pyrimiding and strategic disease resistance management.

As an intracellular biotroph, *P. bassicae* uses phagotrophic ingestion for nutrition from host cells during its infection cycle, while all of the time secreting effector proteins to 'mask' its presence in the cell of its host plant. This pivotal role of pathogen-secreted effector proteins in the establishment, and subsequent progression of clubroot disease makes them the obvious targets for identification of form and *in planta* function and their subsequent exploitation in an *in planta* RNAi approach to engineering pathogen resistance.

The objectives of our study were to:

1. identify the colonisation and disease development transcriptome of *P. brassicae* from large scale EST datasets (40,000) using suppression substractive hybridisation libraries at different disease developmental stages.
2. profile host (Arabidopsis and canola) and pest (*P. brassicae*) gene expression during different disease developmental stages using next generation high throughput sequencing (Illumina). Findings used to develop molecular markers for commercial canola resistance breeding programs.
3. identify *P. brassicae* effector proteins involved in colonization of canola roots and the identification and characterization of *Avr* genes. Cataloging and characterizing prevalent races based on the presence of *Avr* genes triggering a plant hypersensitive response (HR).
4. deploy an *in planta* RNAi approach to impart resistance to clubroot in commercially grown, high-yielding Canadian canola varieties.

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

To enable us to carry out the work proposed above, we had to first establish a clubroot disease nursery, a dual culture system for the pathogen and labeling techniques for *in planta* visualisation of the pathogen.

Establishment of clubroot nursery:

Initially we obtained clubroot tissue infected with *P. brassicae* pathotype 3 from Dr. G. Peng at AAFC, Saskatoon. This was used to infest soil in plant growth trays in a growth chamber (Conviron) located in the Environmental Effects on Plant Pathogen Interactions (EEPPI) centre in the basement of the Biology department. The EEPPI centre, the result of a successful CFI leadership grant application, contains five Conviron growth chambers and space for all plant work and is currently used exclusively for clubroot research. Canola (Westar – susceptible to clubroot disease) is grown in the infected soil inorder to; i) amplify the production of infected canola roots (see Figure 1), from which we isolate *P. brassicae* inoculum required for our work, and ii) maintain a source of infected soil for our current and future research projects and training programs that are related to clubroot disease.

Figure 1. Development of clubroot symptoms on host canola.
Infected clubroots of canola plants grown in the disease nursery photographed at 25 days post inoculation with *P. brassicae*.

Establishment of a dual culture system for studying P. brassicae-host interactions:
To complement the clubroot nursery, we have successfully established a dual culture pathogen-callus system to study *P. brassicae* and host plant cell interactions. In the dual culture pathogen-callus system, we were able to produce a high abundance of zoospores as well as resting spores in callus tissues generated from infected canola clubroots growing on MS plates in the absence of plant growth hormones (Figure 2).

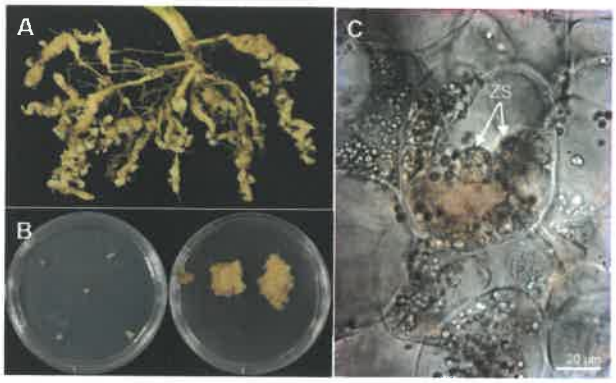

Figure 2. Development of *P. brassicae* in the callus cells generated from infected canola clubroot tissues.
A. Clubroot of *B. napus* grown for 60 days in soil infested with *P. brassicae*.
B. Segments of infected clubroot tissues incubated on plates of regenerating medium (MS plates) minus plant growth hormones for 60 days.
C. Newly generated callus was hand sectioned and examined under a compound microscope. Abundant zoosporangia (ZS) (arrows) in an infected callus cell.

The inoculum obtained from the cells of dual culture calli is pathogenic on canola (Westar) (Figure 3) and Arabidopsis (Col-0 ecotype) (Figure 3) in axenic cultures.

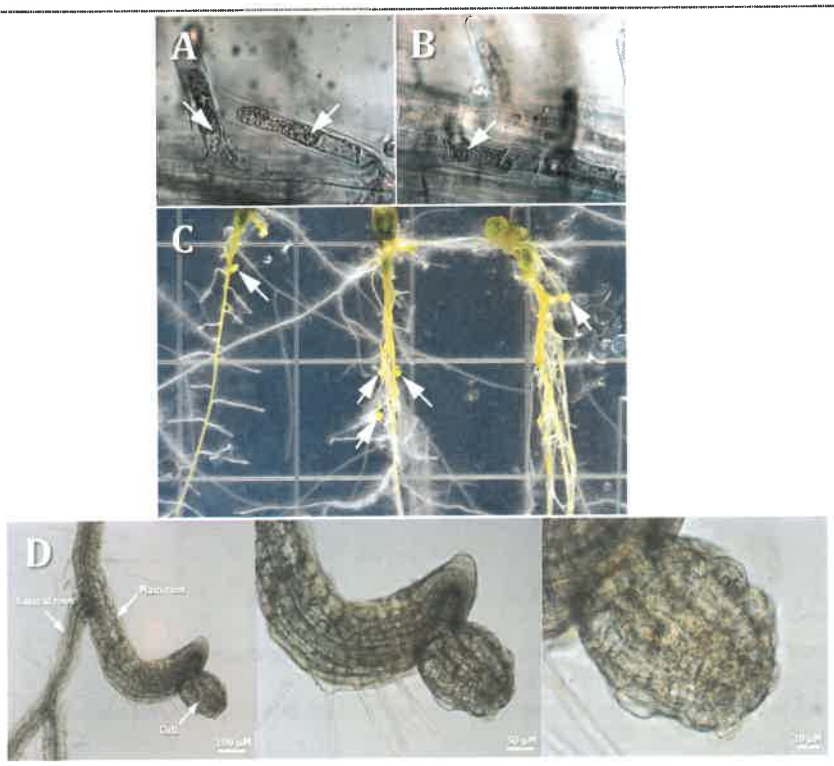

Figure 3. Infection of *B. napus* and Arabidopsis roots with *P. brassicae* inoculum prepared from dual culture
A & B. Inoculated *B. napus* roots at 48 hpi were examined under a light microscope. Both root hairs (A) and epidermal cells (B) were colonized by plasmodia of *P. brassicae*. The host nucleus (arrows) was enlarged, possibly due to endoduplication triggered by *Plasmodiophora* infection.
C. Gall formation on the surface of *B. napus* roots grown on the culture media after inoculation from *B. napus* calli containing *P. brassicae*.
D. Four day-old Arabidopsis seedlings on ½ MS medium plates were inoculated with *P. brassicae*. At 45 dpi, the formation of galls were obvious under light microscopy. The gall appears in the main root elongation zone of the Arabidopsis plant.

The successful establishment of the dual culture system has allowed us to more easily investigate the pathogenesis of the clubroot pathogen within host cells and tissues. Furthermore, the success of the dual culture system provides an alternative source of inoculum for our current and future research projects and training programs that are related to clubroot disease.

Labeling techniques for in vivo visualisation of the intracellular pathogen P. brassicae:
During the infection process, the pathogen develops a series of specialized infection-related structures such as primary/secondary zoospores, primary/secondary plasmodia, zoosporangia, and resting spores that together comprise a complex pathogen life cycle within host tissues. These specialized infection-related structures are very small in size and/or highly variable in shape. From histological studies using TEM and light microscopy, we have shown that, in contrast to *Arabidopsis* or canola root tissues, the infection structures of *P. brassicae* contain abundant lipid bodies (Figure 4).

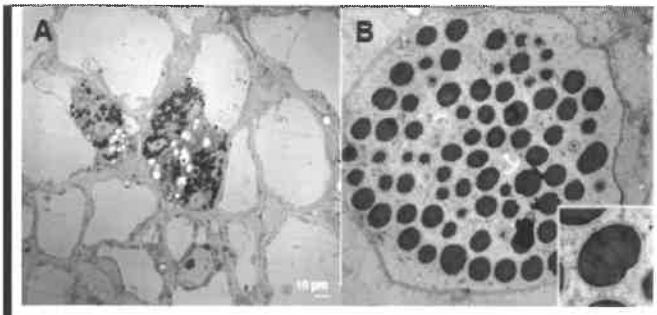

Figure 4. Abundance of lipid droplets at different development stages of *P. brassicae* in Arabidopsis.
A. TEM image revealed presence of plasmodia in cortical cells of infected root at 40 dpi. Lipid droplets were stained with osmium and appear dark.
B. TEM image of an infected cortical cell filled with developing resting spores at 60 dpi. Inset is enlarged image of a resting spore containing numerous lipid droplets.

These histological results led us to investigate whether staining lipid bodies by fluorescence probes would allow for the *in vivo* detection of *P. brassicae* in host tissues during infection processes. Utilising the two lypophilic dyes FM4-64; a living dye known to label the plasma membrane within minutes and the tonoplast after about an hour - it is commonly used as a marker of cell endocytosis since it cannot freely diffuse through the membrane, and Nile red; diffuses freely through the plasma membrane and does not require endocytosis to be internalized into living cells, we were able to differentially stain the various pathogen life-cycle structures (Figures 5&6). FM4-64 was not internalized into plasmodia (Figure 5A) or zoosporangia (Figure 5B&C), however, zoospores released from the zoosporangium were entirely stained by the dye, showing bright red fluorescence (Figure 5B). Unlike FM4-64, Nile red-stained zoospores inside zoosporangia (Figure 7A,B) and also labeled zoospores within the zoosporangia (Figure 7C). We therefore concluded that FM4-64 was not suitable for labeling *P. brassicae* within infected plant cells whereas Nile red is an efficient and effective means of visualising *P. brassicae* structures (through the presence of abundant oil bodies) within infected plant cells.

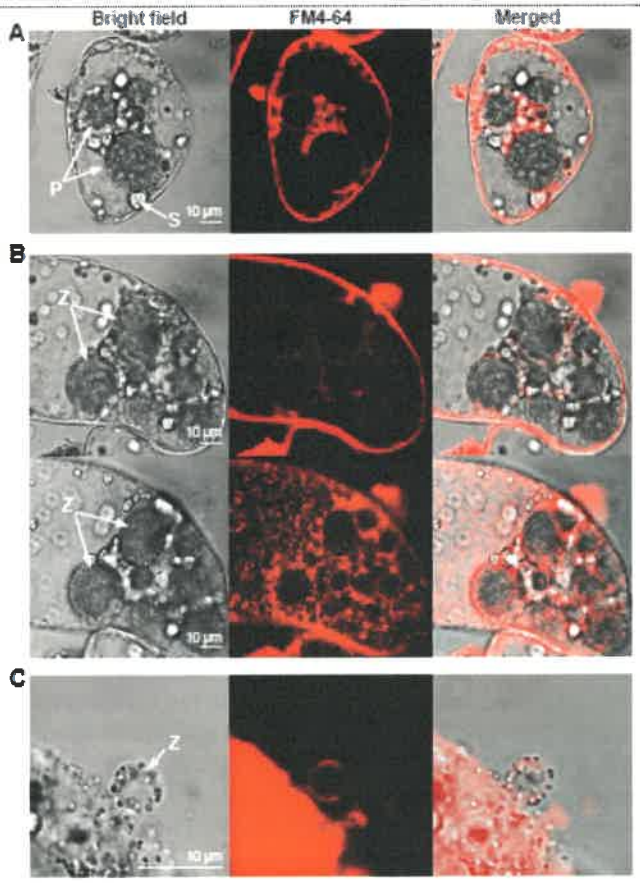

Figure 5. Staining of *P. brassicae* structures in infected canola cells by the lipophilic styryl dye FM 4-64.
Segments of canola clubroot tissues at 30 dpi with *P. brassicae* were surface sterilized and incubated on MS medium plates. At 3 dp incubation, fresh callus tissues were stained with FM4-64 and examined under confocal microscopy.
A. A callus cell with plasmodia stained with FM4-64. FM4-64 stained several compartments of the host callus cell and the periphery of plasmodia, but failed to stain the internal contents of plasmodia.
B. FM4-64 stained zoospores. Upper panels show a callus cell with zoosporangia stained with FM4-64. After an extended incubation on microscope slide for 1 hour, bottom panels show release of zoospores into host cell from zoosporangia.
C. A suspended zoosporangium stained with FM4-64. FM4-64 labelled the periphery of the zoosporangium.
P, plasmodia; S, starch granules; Z, zoosporangia.

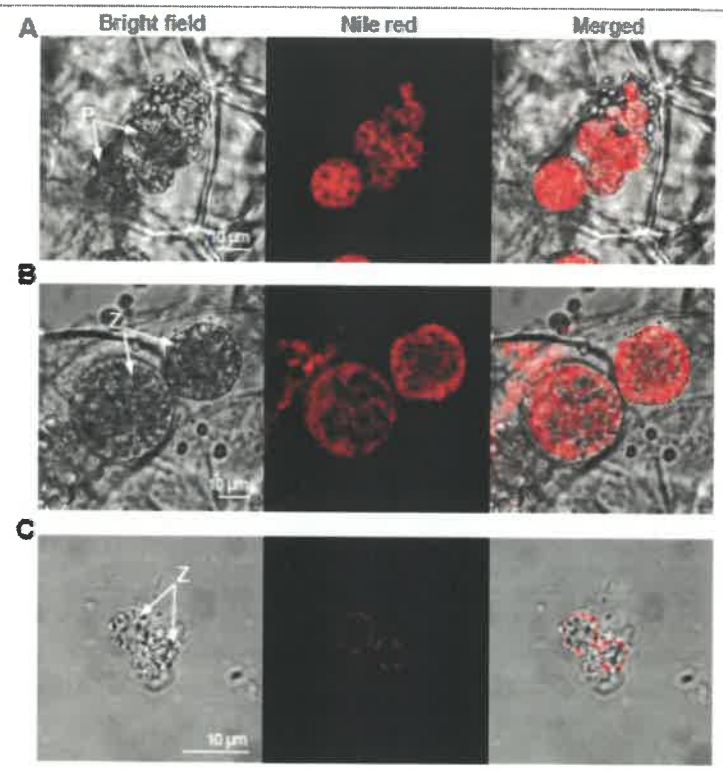

Figure 6. Staining of *P. brassicae* structures in infected canola cells by Nile red.
Segments of canola clubroot tissues at 30 dpi with *P. brassicae* were surface sterilized and incubated on MS medium plates. At 3 dp incubation, fresh callus tissues were stained with Nile red and examined under confocal microscopy.
A. Callus cell with Nile red staining the internal contents of plasmodia. P; plasmodia
B. Nile red stained zoospores within zoosporangia in infected callus cells. Z, zoosporangia
C. Nile red labelled zoospores encased within zoosporangia.

Nile red provides a powerful staining method for monitoring pathogen development inside the host cell during pathogenesis and provides us a tool by which we are able to correlate transcriptome (cDNA) data with stage of infection and disease progression.

Objective 1

cDNA library construction and annotation:

A full length cDNA library from total RNAs of gall tissues of *P. brassicae*-infected canola roots 35 days post inoculation (dpi) was constructed, from which 20,000 clones were isolated and 10482 sequenced (NRC, Saskatoon). The 10482 cDNA library reads were trimmed of vector sequences and filtered for minimum sequencing quality using the phred (http://www.phrap.org/phredphrapconsed.html) and cross_match (http://www.phrap.com/index.htm) programs. This resulted in 9478 high-quality sequences free of any vector fragments. Redundant sequences were removed using the CD-HIT program (http://weizhongli-lab.org/cd-hit) with a 97% identity cut-off. This left 4396 high-quality, unique, trimmed cDNA sequences. These cDNA sequences were then mapped against both a draft *P. brassicae* genome (*P. brassicae* pathotype 3, obtained from Hossein Borhan at AAFC) and the *Brassica napus* genome (as reported in *Science* 345(6199):950-953) using the STAR program (https://code.google.com/archive/p/rna-star). The cDNA sequences were categorised according to the mapping results. 1987 sequences were deemed to be definite *P. brassicae* hits, while 1635 were definite *B. napus* hits and 774 did not map to either organism. All the *P. brassicae* hits and unmapped sequences (total 2761 sequences) were searched using BLASTX against predicted *P. brassicae* protein sequences obtained from Hossein Borhan. The 2761 sequences mapped to 1608 unique protein sequences, which were then used as input to SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) to predict putative secretory proteins. We found a total of 132 putative secretory protein coding cDNA clones that were 'confirmed' by reverse sequencing and manual checking. Of

the 132, 75 cDNA sequences have predicted functional domains and the remaining 57 are unknown.
As a subsequent analysis stage, Blast2GO (https://www.blast2go.com) was used to map GO terms to each of the sequences in the cDNA library. Of the sequences deemed to be from *P. brassicae*, 892 were annotated, while 1567 of the *B. napus* sequences were annotated. Pie charts were generated to show GO terms at levels 2 and 3 for each organism (Appendix 1). GO term enrichment analysis was also performed with Blast2GO using an FDR cut-off of 0.05 and Blast2GO's option to reduce the list of GO terms to the most specific ones. A two-sided Fisher's exact test was used to determine GO terms that were significantly under- or over-expressed for either of the two organisms (*P. brassicae* and *B. napus*).

Objective 2

Gene expression during different disease development stages – RNA-seq:

Total RNAs were extracted from leaves and roots of *P. brassicae*-infected and control Arabidopsis plants at 17, 20 and 24 dpi. The total RNAs were sequenced at NRC, Saskatoon using the Illumina RNA-seq kit for construction of the cDNA libraries and paired-end sequencing was carried out on the Illumina HiSeq 2500 platform. The raw RNA-seq reads were trimmed of adapter sequences and low-quality bases using the Trimmomatic program (http://www.usadellab.org/cms/trimmomatic). The available Arabidopsis genome was used as a reference file (ftp://ftp.ensemblgenomes.org/pub/plants/release-30/fasta/arabidopsis_thaliana) and it was indexed using bowtie2-build (http://bowtie-bio.sourceforge.net/tutorial.shtml#newi). Tophat (https://ccb.jhu.edu/software/tophat/manual.shtml) was used to map the RNA-seq reads against the Arabidopsis reference genome, producing binary alignment/map (BAM) files. The BAM files were used as input to the DESeq2 package (http://www.bioconductor.org/help/workflows/rnaseqGene) for the R programming language (https://www.r-project.org/) to calculate differences in gene expression between treatment (infected plants) and control (uninfected plants) conditions at the different time points. GO analysis was carried out in agriGO (http://bioinfo.cau.edu.cn/agriGO/analysis.php) and MapMan (http://mapman.gabipd.org/web/guest/mapman) was used for identifying and mapping specific pathway expression patterns.

Objective 3

Expression profiling of P. brassicae putative secretory effector proteins:

Semi-quantitative expression patterns of the 75 predicted putative secretory protein coding genes (objective 1) containing predicted functional domains were studied at different time points; 0, 2, 5, 7, 14, 21 and 28 dpi in Arabidopsis Col-0 infected with *P. brassicae* pathotype 3 (Figure 7).

Objective 4

RNAi mediated resistance to P. brassicae:

It is unknown whether *P. brassicae* possesses a RNAi mechanism. At the time of RNAi cassette construction only 113 *P. brassicae* sequences were known. To identify whether a RNAi mechanism is present in *P. brassicae* and that it can be hijacked as a method to inhibit secondary infection and subsequent clubroot disease in canola, transgenic Arabidopsis plants carrying single KO constructs for *PbPRO1* (serine protease, 1404 bp), *PbSRZ2* (putative effector protein, 726 bp) or the double KO construct *PbGST/PbGRX* (Glutathione S-transferase-like, 693 bp / Glutaredoxin, 448 bp) in the constitutive binary vector pKannibal (SnapGene: http://www.snapgene.com/resources/plasmid_files/plant_vectors/pKANNIBAL/) were constructed (Appendix 4). *Agrobacterium tumefaciens* GV310 was used as the carrier for the RNAi constructs. Agrobacterium-mediated transformation of Arabidopsis Col-0 was carried out following the protocol of Zhang *et al* (2006). T0 seeds were plated on kanamycin and PCRs were carried out to confirm the selection of positive T1 transgenic plants. From these, 10 homozygous T2 lines were generated for infection with *P. brassicae*. All plants were grown in the EEPPI centre groth chambers at 23°C, under 16/8 hours light/dark, with 97% humidity and 44 µmol/m^2/s light intensity. A preliminary resistance test was carried out on five plants from each of five positive lines for the double KO *GST-GRX* RNAi construct and wild type Arabidopsis with three replicates of the study. After germination each plant was infected with $2*10^7$ *P. brassicae* zoospores, freshly isolated from clubroot galls soaked in water for 3-4 hr followed by grinding in water and filtering through mira cloth. Non-infected wild type Arabidopsis plants were also used as controls. Roots were cleaned at 14, 21 and 27 days post infection (DPI), scored against a disease index and photographs taken, using a Canon camera (Rebel, 2X), of the whole plant and cleaned roots.

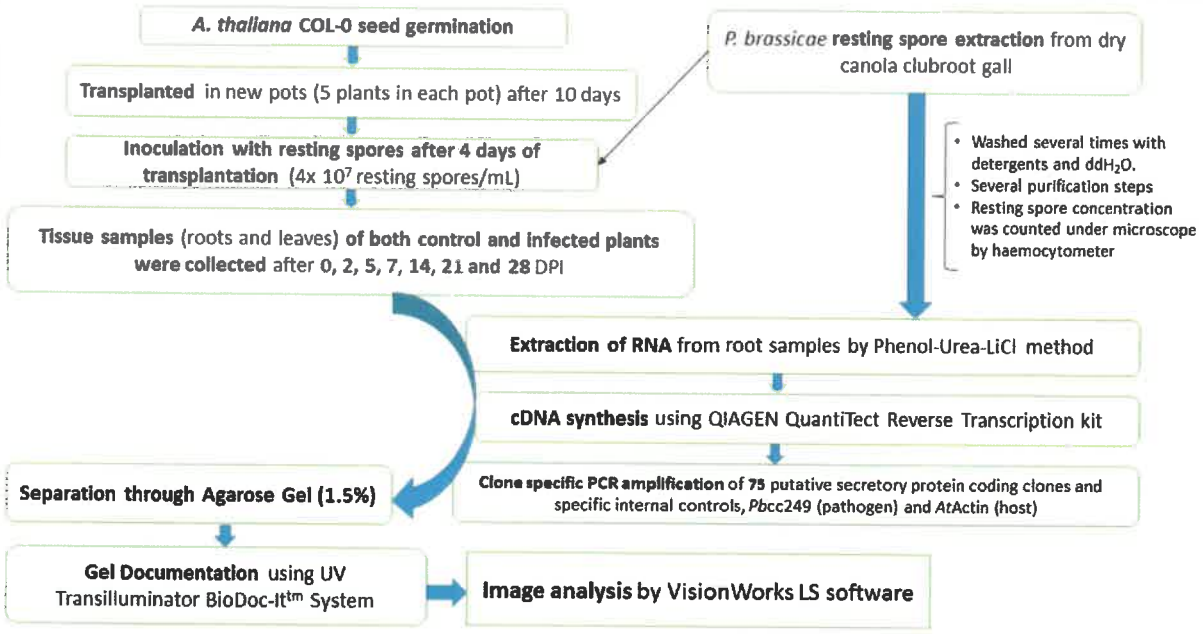

Figure 7: Workflow of the experimental approach to study the expression patterns of *P. brassicae* putative secretory protein genes in *A. thaliana*.

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) **Identifying the colonisation and disease development transcriptome of *P. bassicae* from large scale EST datasets (40,000) using suppression subtractive hybridization libraries at different disease development stages (Years 1&2)**	Best tissue type and time of infection selected and library constructed from 35 dpi clubroot from canola; 20,000 clones isolated and ~10,500 clones sequenced. Resulting sequencing a ~50:50 split between canola and *P. brassicae* sequences. Focused on the *P. brassicae* sequences for the isolation of putative effectors.
2) **Host (Arabidopsis and canola) and pest (*P. brassicae*) gene expression profiling during different disease development stages using next generation high throughput sequencing (Illumina). Findings used to develop molecular markers for**	Transcriptomes generated from 17, 21 and 24 dpi Arabidopsis above and below ground tissues. Comparative analyses of the transcriptomes carried out using AgiGO and MapMan. Above ground transcriptomes were generated in attempt to identify possible 'markers' for rapid disease identification without the need for pulling crops in the field. Molecular markers are yet to be identified, however, the transcriptome data is still

commercial canola resistance breeding programs (Years 1&2)	to be completely analysed.
3) Identification of *P. brassicae* effector proteins involved in colonization of canola roots and the identification and characterisation of avirulence genes. Cataloging and characterizing prevelant races based on the presence of avirulence genes triggering a HR (Years 2&3)	Putative secretory effector proteins identified from *P. brassicae*. Those not expressed in resting spores but expressed during disease progression are of most importance for possible use as RNAi targets. Identification of avirulance genes and characterisation of prevelant races not carried out.
4) Deploying an *in planta* RNAi approach to impart resistance to clubroot in commercially grown, high-yielding Canadian canola varieties (Years 3&4)	Homozygous T2 plants generated carrying a single (two different constructs) or double RNAi constructs. Double construct *PbGST/PbGRX* transgenic lines were tested as proof of principle for RNAi induced resistance to *P. brassicae*. Single construct lines still to be tested.
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.*

Our histological/microscopy techniques have provided us with the ability to view how the pathogen hides itself inside the plant cell. This work is ongoing and continues to be funded through the Growing Forward programme. Being able to visualise and connect the identification of pathogen life cycle and clubroot disease progression with pathogen effector protein gene expression will provide us the information needed for the selection of a second round of targeted RNAi (effector protein) genes. We have identified 15 putative secretory effector protein clones (Appendix 3) that are not expressed in resting spores of *P. brassicae* but are expressed at a variety of later stages of infection (For four examples see Figure 8), suggesting that these genes may be associated with pathogenicity and important during primary and/or secondary infection with *P. brassicae.*

In plants, Programmed Cell Death (PCD) is described as a natural occurance in the hypersensitive response against pathogens and/or other external stimuli (Lamb & Dixon, 1997). During early infection the defence mechanism of a host plant can recognize the presence of the pathogen (by the presence of a pathogen secreted effector protein) and respond with an Effector Triggered Immune (ETI) response-associated Plant Cell Death (PCD) (Dou *et al.* 2008). Of the 15 putative secretory proteins that are not expressed in resting spores, 10 (Table 1) have functional annotations identifying their possible involvement in PCD conceivably through mimicking plant proteins and thereby silencing the PCD process or through manipulating the host ubiquitination pathway (intrinsic to PCD) such that it functions against its own 'pathogen resistance' (*R* gene) proteins (Chisholme *et al*, 2006) - resulting in the *P. brassicae* pathogen being able to hide inside the host plant cell.

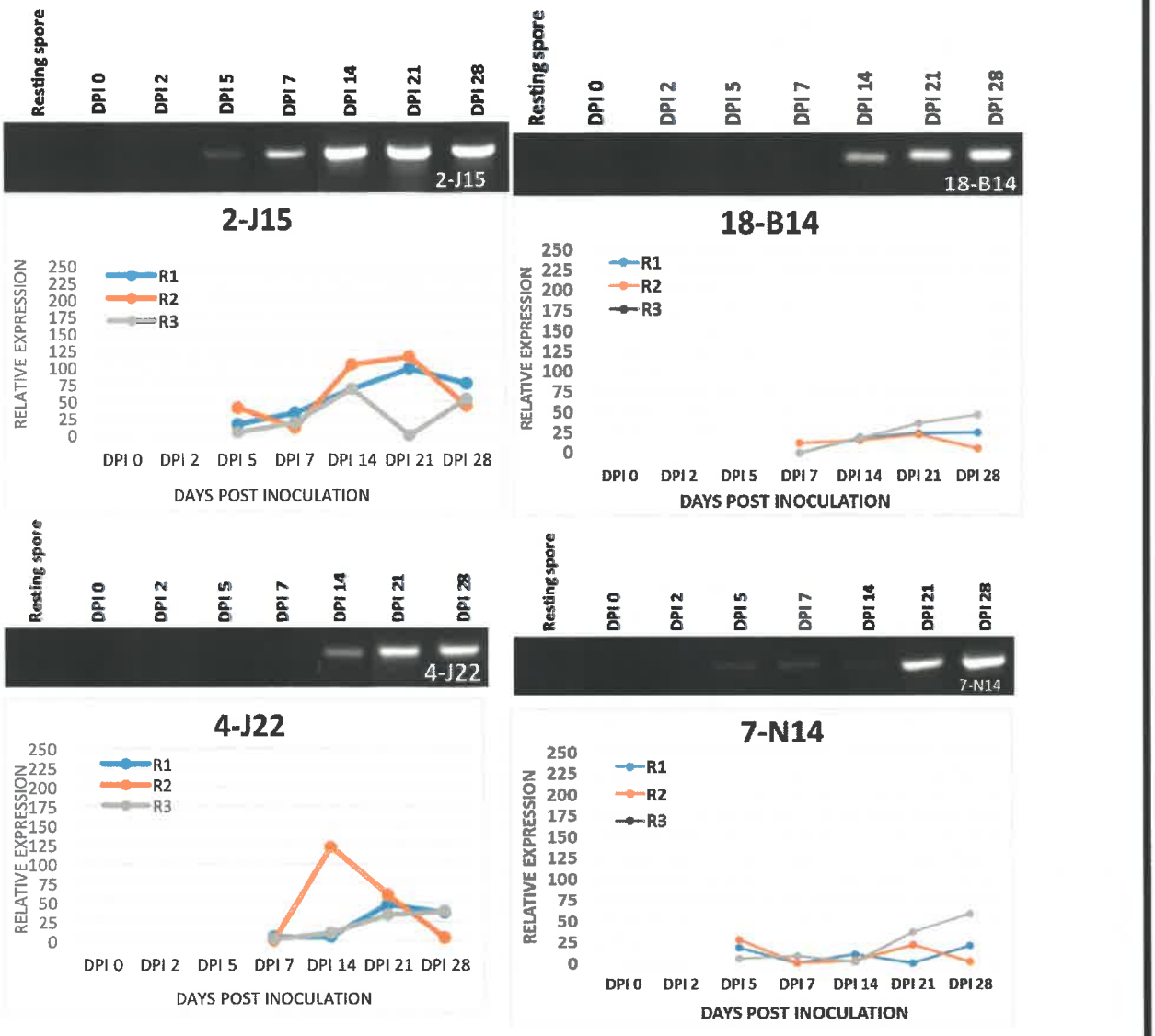

Figure 8: Relative expression of candidate *P. brassicae* putative effectors expressed at different time points. The expression of all *P. brassicae* genes was measured with respect to the expression of the *P. brassicae* internal control (*Pb*cc249) that was set at 100.

Table 1. A list of *P. brassicae* candidate effectors selected for screening for regulatory function against host-triggered PCD.

Clone#	Domain info (CDD search)	BLASTP (nr)	E-value	RS express
16-J02	B-cell receptor-associated protein 31 (Bap31) (pfam05529); Reovirus sigma capside protein (pfam04582)	PREDICTED: LMBR1 domian-containing protein 2 homolog [*Trichogramma pretiosum*]	0.42	Yes
2-J15	Catalytic domain of Protein Kinases (cd00180)	PREDICTED: serine/threonine-protein kinase HT1-like [*Setaria italica*]	3e-16	No
6-J07	pfam01169: Uncharacterized protein family UPF0016 and COG2119: Putative Ca2+/H+ antiporter	PREDICTED: Transmembrane protein 165-like [*Hydra vulgaris*]	3e-72	No
4-J22	(SUI2) Translation initiation factor 2, alpha subunit (eIF-2α) (COG1093)	Not found	-	No
23-J17	Ankyrin repeat-4 (pfam13637) and Ankyrin repeat-2 (pfam12796)	PREDICTED: receptor-interacting serine/threonine protein kinase HT1-like [*Setaria italic*]	2e-19	No
27-H14	U-box/Modified RING finger domain (smart00504)	PREDICTED: U-box domain-containing protein 51-like [*Camelina sativa*]	0.03	No
7-N14	No domain info available	Ubox domain containing protein [*Acanthamoeba castellanii* str. Neff]	0.11	No
21-C06	DUF86 superfamily and Med-3 domain	Not found	-	No
18-B14	No domain info available	Not found	-	No
23-A03	No domain info available	Not found	-	No
20-J15	No domain info available	Cystatin [*Lepidoglyphus destructor*]	1e-10	No info

A graduate student has taken on this project for their PhD thesis; 'Identification and functional characterisation of putative effectors of *Plasmodiophora brassicae* and their role in regulating cell death during infection'. Confirmation that this 'hiding' mechanism is integral to the life cycle of the pathogen and subsequent disease progession will provide us with a target for a structured establishment of improved clubroot resistance in canola.

The transcriptomes from the 17, 20 and 24 dpi above and below ground infected (and control) tissues are still being analysed and concurrently a manuscript highlighting the major and significant differences in networked responses to infection is being prepared. We have included initial MapMan analyses below (Figure 9A&B) showing the complexity of the data, but also how it can be network-analysed. The recent publication of the *P. brassicae* genome sequence (Schwelm *et al*, 2015) and access to the *P. brassicae* genome data in AAFC (Hossein Borhan's lab) has provided the reference genome required for these types of analyses.

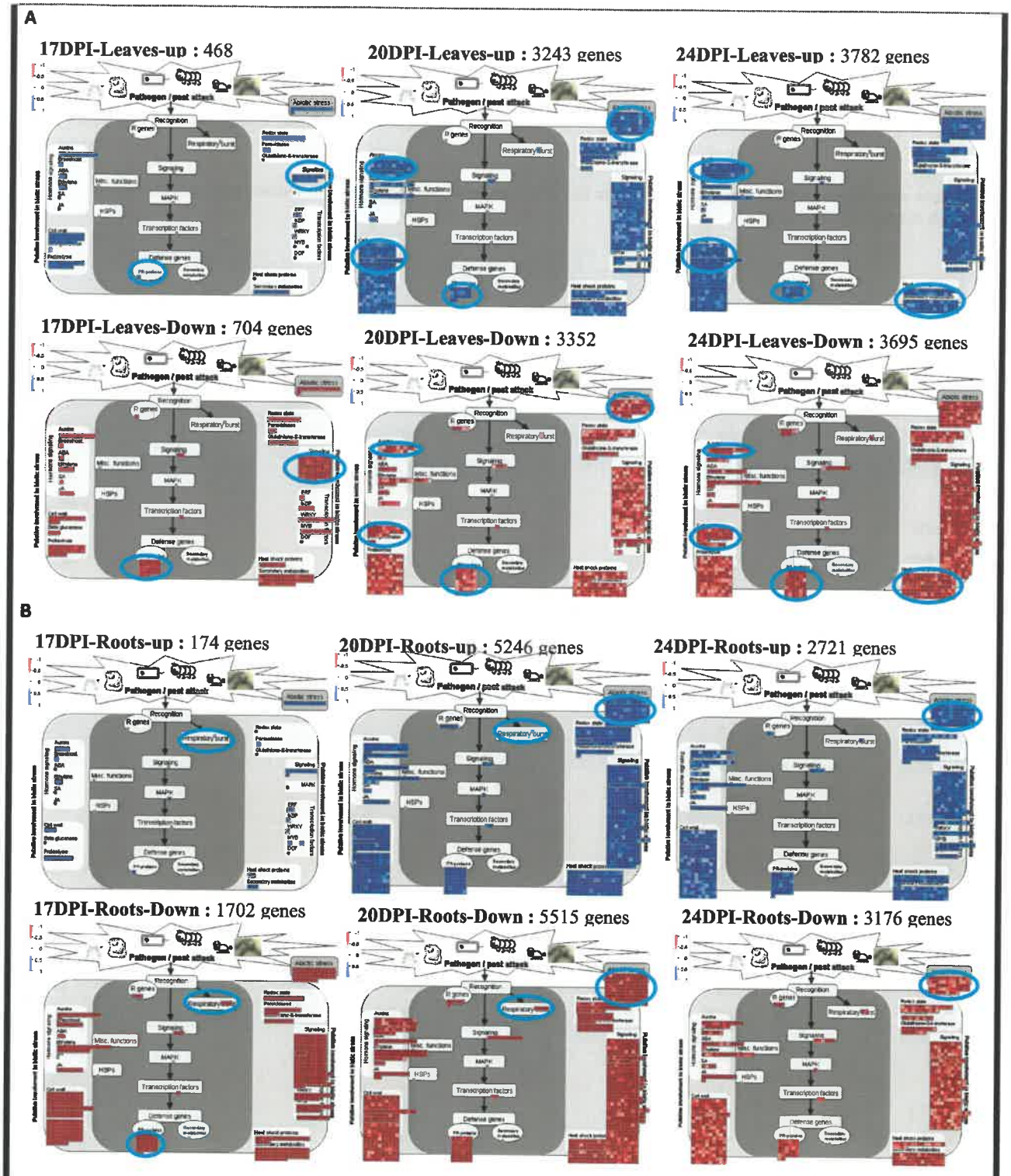

Figure 9: Network analyses (MapMan) showing changes in relative expression of the transcriptomes of 17, 20 and 24 dpi (*P. brassicae* pathotype 3) above and below ground Arabidopsis tissues relative to control uninfected tissues.

Initially, without access to the *P. brassicae* genome or any putative secretory effector protein sequences (generated in this study) we decided to use the available sequences for *PbPRO1* (serine protease), *PbSRZ2* (putative effector protein), *PbGST* (Glutathione S-transferase-like) and *PbGRX* (Glutaredoxin) in our RNAi work. A preliminary challenge of the double KO *GST-GRX* RNAi construct and wild type Arabidopsis to *P. brassicae* infection suggests that disease progress in RNAi lines was slower compared to that observed in wild type infected plants, especially for transgenic line 1 (green; Table 2).

Table 2. Root disease index after *P. brassicae* infection for *GST-GRX* RNAi lines, wild type infected and wild type un-infected Arabidopsis plants at 14, 21 and 27 dpi.

	Rep1					Rep2					Rep3				
14DPI	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
Line 1	0	1	1	1	0	0	1	1	1	1	1	1	1	1	1
Line 3	0	0	1	1	1	0	0	1	1	1	1	0	0	0	0
Line 4	0	0	0	1	1	0	0	0	0	1	1	1	0	0	0
Line 10	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0
Line 12	0	0	0	1	0	1	0	1	1	0	0	1	1	1	1
WT (Infected)	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1
WT (Un-infected)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21DPI															
Line 1	2	2	1	0	3	0	0	1	1	0	2	2	2	2	1
Line 3	1	2	1	2	2	1	1	1	1	2	0	2	2	1	0
Line 4	1	2	2	0	2	1	1	1	2	2	2	2	2	1	1
Line 10	1	2	2	0	0	2	2	2	2	2	2	2	2	2	2
Line 12	3	3	3	2	2	2	2	2	2	1	3	2	2	1	1
WT (Infected)	3	3	2	2	1	3	3	3	3	2	3	2	2	1	1
WT (Un-infected)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
27DPI															
Line 1	2	2	2	3	2	2	2	2	2	2	2	2	2	2	2
Line 3	4	1	2	3	3	5	2	2	3	0	2	2	1	1	0
Line 4	4	4	4	0	4	3	3	3	4	3	3	3	3	4	0
Line 10	2	2	3	3	5	1	2	2	3	3	5	5	4	3	3
Line 12	2	2	2	3	3	2	3	4	4	4	2	2	2	2	1
WT (Infected)	5	5	5	5	5	3	3	4	2	2	3	3	3	3	2
WT (Un-infected)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Further pathogen challenges need to be carried out on these double construct RNAi lines as well as the single construct lines (yet to be tested) and future identified secretory protein effector constructs to confirm this outcome.

Setbacks:

There were a number of personnel changes during the course of this project and a six-month extension of the project was approved to July 31, 2015.

1. The first PDF on the project (who produced a cDNA library of unusable sequences) was terminated Aug, 2012 with his replacement not starting until late Oct 2012. At this point the gall cDNA library was constructed. This set us back approximately 6-9 months on achieving our goals with regards to the RNAi lines and utilising the cDNA sequences obtained from the cDNA library.
2. Jiangying Tu (PhD student) was on maternity leave between Feb-Oct 2014.
3. Analyses of the transcriptome data has proven more difficult than expected and external help was obtained through Dr. Tony Kusalik, Department of Computer Sciences, and his graduate students and post-doc, Dr. Brett Trost.

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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.*

Through this ADF project, we have:

- developed a highly sensitive, and specific *in planta* detection method that enables us to visualize the *P. brassicae* infection-structures during pathogenesis. The technique will facilitate the investigation of the pathogen life cycle in further details, to examine the host cellular responses during clubroot pathogenesis, and to determine the cellular mechanisms of resistance among canola varieties against clubroot disease,
- transcriptome data for infected and control, above and below ground tissues, that is still being analysed but will provide the understanding of the whole plant networked response to infection, together with the pathogen effector profile at various stages of infection and disease progression,
- initial RNAi trial results that suggest the possibility of utilising this technology in the establishment of improved resistance to clubroot in canola.

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.*

Visualisation technologies - aid to researchers in the future linking of the *P. brassicae* life-cycle with disease progression in the canola root – understanding the disease will better inform resistance strategies.
The transcriptome and cDNA data are still being analysed, however, a student is currently looking into the possible role of putative secretory effector proteins in enabling the pathogen to hide from the plant PCD process. These effectors could be possible targets for the production of future RNAi resistance lines.

The results from this work are still at an early stage of 'usefulness' to the producer/industry but will be of great use to the clubroot research community.

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

To date, no commercialized products have resulted from this research, however:

1. Patent: Yangdou Wei and Cindy Zou, **Use of Glyphosate to Control Clubroot Disease,** Application Number: 14/622889; Publication Date: 08/20/2015,
2. the sequences and clones generated from the cDNA library and the transcriptome sequencing data will be released to the research community as a condition of publication of the manuscripts currently in preparation,
3. the transgenic RNAi lines will be available for research once we have followed up on our preliminary results,
4. the pathogen visualisation techniques will be available for research once the manuscript currently in preparation is published.

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

Conference presentations:

1. **T Missihoun***, Y Wei & PC Bonham-Smith (2015) Use of *Brachypodium distachyon* as a non-host species to find resistance against clubroot disease in Brassicacea, Botany 2015, Edmonton, Canada. (ADF# 20130096.)
2. **J Tu***, Y Wei & PC Bonham-Smith (2015) Alteration of PIN-mediated auxin transport modulates clubroot disease symptom development during Arabidopsis-*Plasmodiophora* interaction, Botany 2015, Edmonton, Canada.
3. **MMd Hossain*, IS Sheoran, CS Stewart**, Y Wei & PC Bonham-Smith (2015) Identification of *Plasmodiophora brassicae* genes expressed during infection of *Arabidopsis thaliana* Col-0, Botany 2015, Edmonton, Canada.
4. **CS Stewart***, Y Wei & PC Bonham-Smith (2014) Gene ontology analysis of *Plasmodiophora brassicae* pathogenesis in *Brassica napus*, 9th Canadian Plant Biotechnology Conference, Montreal.
5. **Stewart CS***, Wei Y & Bonham Smith PC (2013) Molecular insight into *Plasmodiophora brassicae* pathogenesis in *Brassica napus*, 2013 International Clubroot Workshop, Edmonton, Alberta; 2013 Canadian Phytopathological Society Annual Meeting, Edmonton, Alberta.

Articles in the news:

1. Understanding the cellular mechanisms of clubroot disease and developing a new form of clubroot resistance. *Canola Digest Science 2015*: http://www.canolacouncil.org/media/573974/canola_digest_science_2015/index.html#30 Highlighting an alternative approach to clubroot disease.
2. Big help from a small grass? The quest for clubroot resistance goes to an unusual source. (2014) *Agannex*: https://www.agannex.com/energy/big-help-from-a-small-grass (ADF# 20130096). Highlighting Tagnon Missihoun's work on clubroot resistance in *Brachypodium distachyon*.
3. New tools help battle canola disease. (2013) *Young Innovators series, a U of S initiative in partnership with the Saskatoon StarPhoenix*. Highlighting Jiangying Tu's work on clubroot in canola.

13. List any industry contributions or support received.

1. The Environmental Effects on Plant Pathogen Interactions (EEPPI) centre (Dept. Biology), obtained through a successful Canada Foundation for Innovation LOF application (2012), is now fully operational and supporting plant growth and experimentation for this project.
2. Funding ($15K/yr) through the Growing Forward 2 program, *The host-pathogen interaction of Plasmodiophora brassicae and canola*, 2014-17.
3. The successful ADF grant, *'Using of non-host species to identify novel genes for durable clubroot resistance in*

canola' started in January, 2014.

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

The genome-wide identification of *P. brassicae* effectors will allow us to decipher the molecular and cellular mechanisms underlying this unique biotrophic, intarcellular host - *P. brassicae* interaction. Identified effector sequences will also allow us to possibly discover *P. brassicae Avr* genes, and to study *P. brassicae* diversity and pathotypes among the infested areas of prairie farm lands.

Based on sequence analysis, expression profiling and gene ontology, we have selected dozens of targeted *P. brassicae* effectors for investigating/characterization of their biological functions. We are aiming to elucidate their roles in clubroot disease development, and to further identify potential host targets.

Three manuscripts are in various stages of preparedness for submission for publication. We hope to see all three submitted and accepted for publication by the end of 2016.

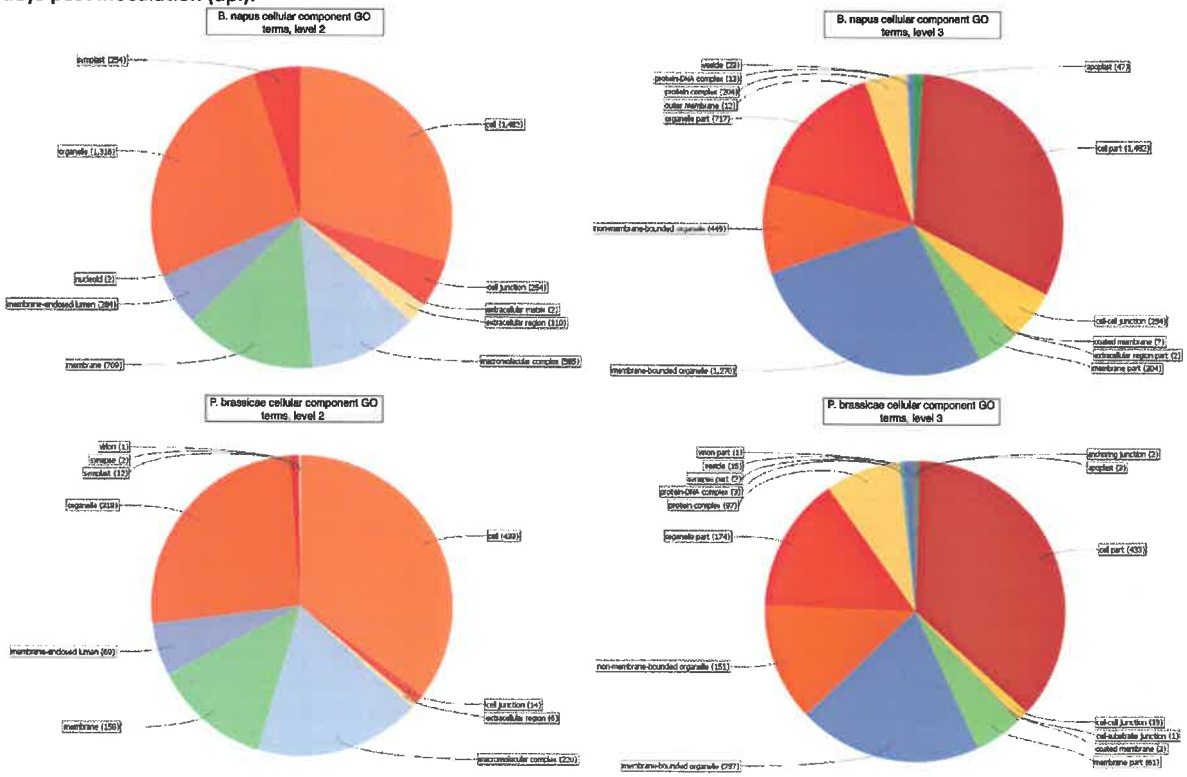
15. Acknowledgements. *Include actions taken to acknowledge support by the Ministry of Agriculture and the Canada-Saskatchewan Growing Forward 2 bilateral agreement.*

All presentations and news articles concerning work from the UofS clubroot research group has and will continue to fully acknowledge the support of the Saskatchewan Ministry of Agriculture and the Canada-Saskatchewan Growing Forward 2 bilateral agreement, as well as the support of the Saskatchewan Canola Development Commission.

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

Appendix 1:

GO annotation of the cleaned up cDNA sequences obtained for *P. brassicae* and *B. napus* from gall tissues of infected canola roots 35 days post inoculation (dpi).



Appendix 2:

RT-PCR expression profiles of 75 putative *P. brassicae* secretory protein coding genes. (+) and (-) indicates the presence and absense of *P. brassicae* genes at particular DPI during infection, respectively.

Clone#	cDNA clone ID	Resting Spore	DPI 0	DPI 2	DPI 5	DPI 7	DPI 14	DPI 21	DPI 28
1	9-M12	-	-	-	+	+	+	+	+
2	25-L07	-	-	-	+	+	+	+	+
3	2-J15	-	-	-	+	+	+	+	+
4	23-A03	-	-	-	+	+	+	+	+
5	7-N14	-	-	-	+	+	+	+	+
6	1-I11	-	-	-	+	+	+	+	+
7	18-B14	-	-	-	-	+	+	+	+
8	4-J22	-	-	-	-	+	+	+	+
9	6-J07	-	-	-	-	-	+	+	+
10	27-H14	-	-	-	-	-	+	+	+
11	24-K22	-	-	-	-	-	+	+	+
12	23-J17	-	-	-	-	-	-	+	+
13	21-C06	-	-	-	-	-	-	+	+
14	9-O12	-	-	-	-	-	-	+	+
15	2-G09	-	-	-	-	-	-	-	+
16	18-H10	+	-	-	-	-	-	+	+
17	22-H09	+	-	-	-	-	-	+	+
18	16-C01	+	-	-	-	-	-	+	+
19	15-G10	+	-	-	-	-	-	+	+
20	20-G19	+	-	-	-	-	-	+	+
21	16-E23	+	-	-	-	-	+	+	+
22	22-B13	+	-	-	-	-	+	+	+
23	12-E05	+	-	-	-	-	+	+	+
24	23-A14	+	-	-	-	+	+	+	+
25	14-M07	+	-	-	-	+	+	+	+
26	4-H14	+	-	-	-	+	+	+	+
27	21-D13	+	-	-	+	+	-	+	+
28	18-G17	+	-	-	+	+	-	+	+
29	3-L22	+	-	-	+	-	+	+	+
30	17-C03	+	-	-	+	+	+	+	+
31	10-I09	+	-	-	+	+	+	+	+
32	18-P03	+	-	-	+	+	+	+	+
33	9-J17	+	-	-	+	+	+	+	+
34	23-B05	+	-	-	+	+	+	+	+
35	26-J15	+	-	-	+	+	+	+	+
36	13-J09	+	-	-	+	+	+	+	+
37	8-N17	+	-	-	+	+	+	+	+
38	15-P03	+	-	-	+	+	+	+	+
39	7-D19	+	-	-	+	+	+	+	+
40	24-M03	+	-	-	+	+	+	+	+
41	18-N15	+	-	-	+	+	+	+	+
42	2-K22	+	-	-	+	+	+	+	+
43	25-J09	+	-	-	+	+	+	+	+
44	9-J22	+	-	-	+	+	+	+	+
45	23-B01	+	-	-	+	+	+	+	+
46	1-C05	+	-	-	+	+	+	+	+
47	2-A05	+	-	-	+	+	+	+	+

48	23-P16	+	-	-	+	+	+	+	+
49	2-E16	+	-	-	+	+	+	+	+
50	9-B01	+	-	-	+	+	+	+	+
51	6-G21	+	-	-	+	+	+	+	+
52	1-P19	+	-	-	+	+	+	+	+
53	9-L13	+	-	-	+	+	+	+	+
54	2-L07	+	-	-	+	+	+	+	+
55	H12-gallcDNA-96	+	-	-	+	+	+	+	+
56	7-J04	+	-	-	+	+	+	+	+
57	27-E04	+	-	-	+	+	+	+	+
58	27-L20	+	-	-	+	+	+	+	+
59	19-H01	+	-	-	+	+	+	+	+
60	9-B11	+	-	-	+	+	+	+	+
61	2-B06	+	-	-	+	+	+	+	+
62	13-C13	+	-	-	+	+	+	+	+
63	5-J11	+	-	+	+	+	+	+	+
64	23-N18	+	-	+	+	+	+	+	+
65	1-P13	+	-	+	+	+	+	+	+
66	2-J08	+	-	+	+	+	+	+	+
67	6-G01	+	-	+	+	+	+	+	+
68	22-M03	+	-	+	+	+	+	+	+
69	8-D14	+	-	+	+	+	+	+	+
70	9-B02	+	-	+	+	+	+	+	+
71	1-K14	+	-	+	+	+	+	+	+
72	1-H02	+	-	+	+	+	+	+	+
73	16-J02	+	-	+	+	+	+	+	+
74	2-F21	-	-	-	-	-	-	-	-
75	20-F08	-	-	-	-	-	-	-	-

Appendix 3:
List of *P. brassicae* candidate effector proteins not expressed in resting spores.

Clone#	Domain info (CDD search)	BLASTP (nr)	E-value*	Expression in resting spores
16-J02	B-cell receptor-associated protein 31 (Bap31) (pfam05529); Reovirus sigma C capsid protein (pfam04582)	PREDICTED: LMBR1 domain-containing protein 2 homolog [*Trichogramma pretiosum*]	0.42	Expression detected
2-J15	Catalytic domain of Protein Kinases (cd00180)	PREDICTED: serine/threonine-protein kinase HT1-like [*Setaria italica*]	3.00E-16	Expression not detected
9-O12	SnoaL-like polyketide cyclase (pfam07366); Predicted ester cyclase (COG5485)	ester cyclase [*Streptomyces kanamyceticus*]	8.00E-10	
6-J07	pfam01169: Uncharacterized protein family UPF0016 and COG2119: Putative Ca2+/H+ antiporter	PREDICTED: Transmembrane protein 165-like [*Hydra vulgaris*]	3.00E-72	
4-J22	(SUI2) Translation initiation factor 2, alpha subunit (eIF-2α) (COG1093).	Not found	-	
23-J17	Ankyrin repeat-4 (pfam13637) and Ankyrin repeat-2 (pfam12796)	PREDICTED: receptor-interacting serine/threonine-protein kinase 4-like, partial [*Octopus bimaculoides*]	2.00E-19	
27-H14	U-box/ Modified RING finger domain (smart00504)	PREDICTED: U-box domain-containing protein 51-like [*Camelina sativa*]	0.03	
7-N14	No domain info available	U-box domain containing protein [*Acanthamoeba castellanii* str. Neff]	0.11	
1-I11	No domain info available	6,7-dimethyl-8-ribityllumazine synthase [*Pseudozyma antarctica* T-34]	9.00E-08	
21-C06	DUF86 (pfam01934) and Med-3 domain (pfam11593)	Not found	-	
9-M12	No domain info available	Not found	-	
25-L07	No domain info available	Not found	-	
24-K22	No domain info available	Not found	-	
18-B14	No domain info available	Not found	-	
23-A03	No domain info available	Not found	-	

Appendix 4:

Table 1. Compilation of common name, primers, fragment size and restriction enzyme information for the three RNAi constructs.

Common name	Primer name	Primers	Size (bp)	Restriction Enzyme
Glutathione S-transferase-like (GST)	GST2-Sense-F	GCGCTCGAGGATAAGACCATCCTGAACG	693	XhoI, EcoRI
	GST2-Sense-R	GCGGAATTCACTTGGCGGGCATGGCGGC		
	GST2-Anti-F	GCGGCTAGAGATAAGACCATCCTGAACG		XbaI, BamHI
	GST2-Anti-R	GCGGGACCCACTTGGCGGGCATGGCGGC		
Glutaredoxin (GRX)	GRX-Sense-F	GCGGAATTCATTTCGTTGGCGGGGACGACG	447	EcoRI, KpnI
	GRX-Sense-R	GCGGGGACCCAAACACTAGGTGTGTGTG		
	GRXAnti-F	GCGGGATCCAGGTCGGTGACGTGGACGACG		BamHI, ClaI
	GRXAnti-R	GCGATCGATCAAACACTACGGGTGTGTG		
Serine Protease (PR01)	PRO1-Sense-F	GCG CTCGAG AGCGCGTGCACATCGGCAGG	1404	XhoI, KpnI
	PRO1-Sense-R	GCG GGTACC CATAGTGTCGCATCGATGACTCGC		
	PRO1-Anti-F	GCG TCTAGA AGCGCGTGCACATCGGCAGG		XbaI, HindIII
	PRO1-Anti-R	GCG AAGCTT CATAGTGTCGCATCGATGACTCGC		
Putative Effector (*Sporisorium reilianum*) (SRZ2)	SRZ2-Sense-F	GCGCTCGAG ATGCTGCGCATGCTGCTTCTGGCCG	726	XhoI, KpnI
	SRZ2-Sense-R	GCG GGTACC CTAGGAACGTTCTTTCAGGTCGGCC		
	SRZ2-Anti-F	GCG TCTAGA ATGCTGCGCATGCTGCTTCTGGCCG		
	SRZ2-Anti-R	GCG AAGCTT CTAGGAACGTTCTTTCAGGTCGGCC		