In-depth Summary for CARP Project 2013-12

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The overarching goal of this research is to identify novel defense genes and underlying molecular framework of the defense response in canola to i) sclerotinia infection ii) application of the biocontrol bacterium PA23 in the presence and absence of sclerotina and iii) identification of cell-specific defense molecules in tolerant and susceptible canola cultivars to sclerotinia infection.

There were 3 main biological questions that were asked during this research:

1. How does global gene expression change during *Sclerotinia* whole-leaf infection in susceptible (Westar) versus tolerant (Zhongyou821) cultivars of canola?

- 1a. what are the major biological processes that contribute to the tolerant phenotype of *Brassica napus* cv. Zhongyou821?

- 1b. what regulatory molecules and transcriptional circuits are responsible for regulating biological processes underpinning tolerance to *Sclerotinia*?

2. What are the tissue-specific defense responses to *Sclerotinia* infection that occur in susceptible versus tolerant cultivars?

3. What changes in gene expression and physiology occur in canola in response to the biocontrol bacterium PA23 in the presence and absence of *S. sclerotiorum*?

Detailed findings from each of these three questions/aims is provided in the following sections:

Aim 1. To identify changes in global gene expression in susceptible versus tolerant canola cultivars of in response to Sclerotinia infection (whole leaf analysis).

Research Personnel – Mr. Ian Girard, MSc student.

Experimental design

- We used next generation RNA sequencing on control and infected leaves of the tolerant *B. napus* genotype ZY821, and the susceptible Westar using a petal inoculation technique to accurately mimic field conditions of Sclerotinia infection,

- We used a robust bioinformatics pipeline to translate the raw RNA-sequencing reads into meaningful biological data. Briefly:
    - The raw reads were filtered for quality control, aligned to the *B. napus* genome using the Tophat suite of alignment, and the Cufflinks package was used to detect new genes and quantify transcript accumulation levels
    - Differential expression analysis and fuzzy k-means clustering was used to identify biologically relevant sets of genes
    - Gene ontology enrichment was performed to identify biological processes active within gene sets
    - Regulatory transcriptional circuits were generated using CanEnrich software to explore how plant defense processes are controlled and identify putative regulatory molecules.

- We then functionally characterized novel defense molecules using T-DNA insertion knockdown lines in a Sclerotinia pathogenicity assay in Arabidopsis.

Key Findings:

- Both susceptible and control cultivars undergo large-scale transcriptional reprogramming following foliar infection of Sclerotinia using the petal inoculation method. ZY821 exhibits a higher level of transcriptional control than Westar; fewer genes were detected overall, but a much greater proportion are highly expressed.

- We discovered over 1200 transcripts with no previous annotation, including genes involved with plant defense processes and signal transduction.

- Using differential expression and gene ontology enrichment software, we identified defense hormone pathways including jasmonic acid and ethylene signalling processes that were enriched in both cultivars after infection. However, we also identified differences in amplitudes between the two cultivars, highlighting the complexity of the interactions.

- Gene that were specifically differentially expressed in the tolerant ZY821 following infection were enriched for protein and chromatic modification processes as well as subcellular trafficking processes and the structural component of the cytoskeleton. We also discovered two putative transcriptional networks active within this set of genes. Transcription factors including *C-TERMINAL DOMAIN PHOSPHATASE-LIKE 3*

(*BnaC02G27760D*), and MYB family genes were predicted to bind to the CCA1 and KAN DNA promoter motifs found upstream of genes enriched for histone methylation and protein modification. We also identified four WRKY DNA-BINDING PROTEINS (WRKYs) predicted to control the plant response to chitin.

- Fuzzy k-means clustering analysis identified ten dominant patterns (DPs) of co-expressed genes with distinct mRNA accumulation patterns. Within DP2, a set of mRNAs accumulating primarily in infect ZY821, we identified a large putative transcriptional module where Heatshock Factors, Ethylene Response Factors (ERFs), and MYC transcription factors act together to control biological processes including defense and redox homeostasis regulatory processes.

- In addition to the ERFs identified in the module analysis, an overall upregulation of ERFs in the tolerant line highlights their roles as mediators of defense to necrotrophic pathogens like Sclerotinia.

- Susceptible phenotypes of Arabidopsis plants compromised in redox homeostasis processes highlights critical role of adaptation to physiological challenges associated with *S. slcerotiorum* infection.

Take home messages

Our rigorous analysis of the RNA-seq data provides novel insight into the transcriptional regulation of complex gene networks underpinning *B. napus* defense to *S. sclerotiorum*.

- Using RNA sequencing we identified previously undiscovered genes associated with the plant defense response.

- New biological processes not previously associated with this pathosystem including protein modification and epigenetic control may contribute to explanation of tolerant phenotype.

- We identified novel regulators of the plant defense processes responsible for providing tolerance to *S. sclerotiorum*. This includes predicted regulators of redox homeostasis, as well as genes previously identified as responsible for defense to *S. sclerotiorum* including homologues of *INDOLE GLUCOSINOLATE METHYLTRANSFERASE 5*, a potential quantitative trait locus for resistance.

Figures

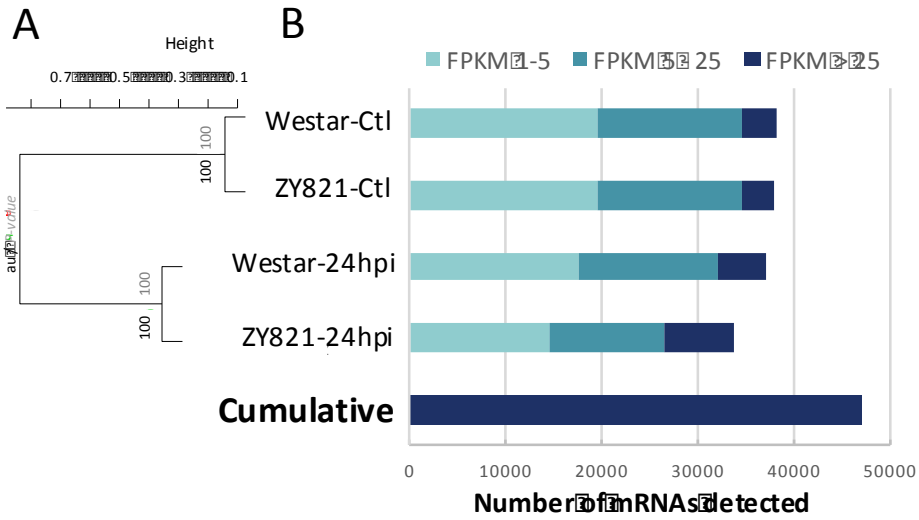

Figure 1.1. RNA Sequencing of susceptible (Westar) and tolerant (ZY821) genotypes of Brassica napus infected with Sclerotinia sclerotiorum using the petal inoculation technique. (A) Hierarchical clustering of genes detected with a minimum of 1 Fragment Per Kilobase of transcript per Million mapped reads (FPKM), with approximately unbiased values in black and bootstrap P-values in grey. (B) Distribution of transcript abundances in FPKM of the four treatments.

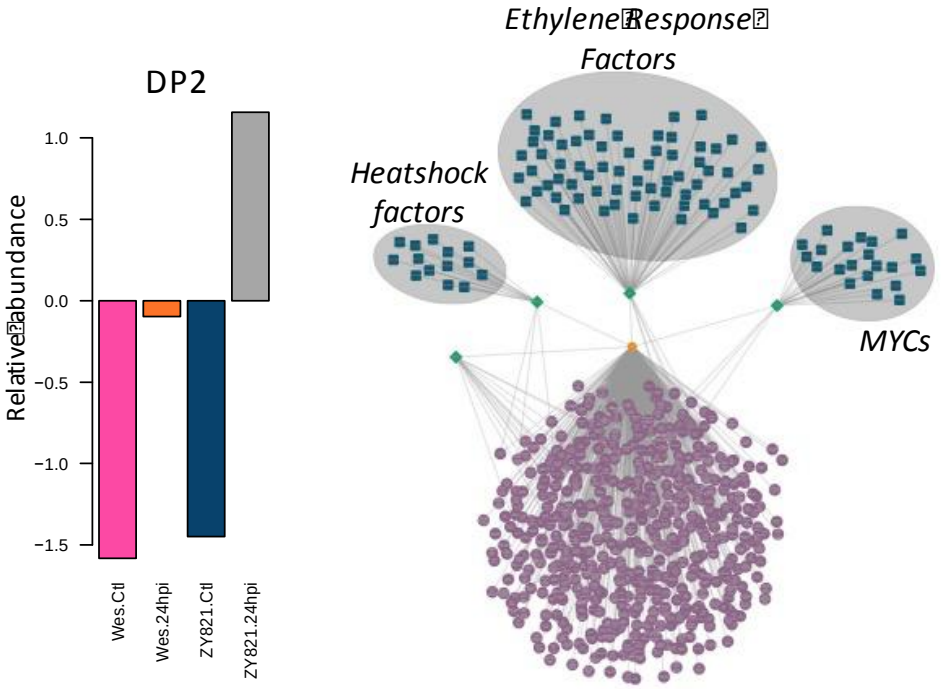

Figure 1. 2 Dominant patterns (DPs) of gene activity discovered using Fuzzy k-means clustering and enrichment analysis. (A) Bar plots showing relative abundance of mRNAs assigned to a DP accumulating primarily in infected ZY821 leaf tissues. (B) Predicted transcriptional module identified in DP2 (gold hexagon) accumulating primarily in Brassica napus cv. ZY821 24hpi. Sets of Heatshock factors (HSF), Ethylene response factors (ERF) and MYC transcription factors (blue squircles) are predicted to bind to their overrepresented DNA motifs (p<0.001) up stream of genes enriched for gene ontology terms (purple circles).

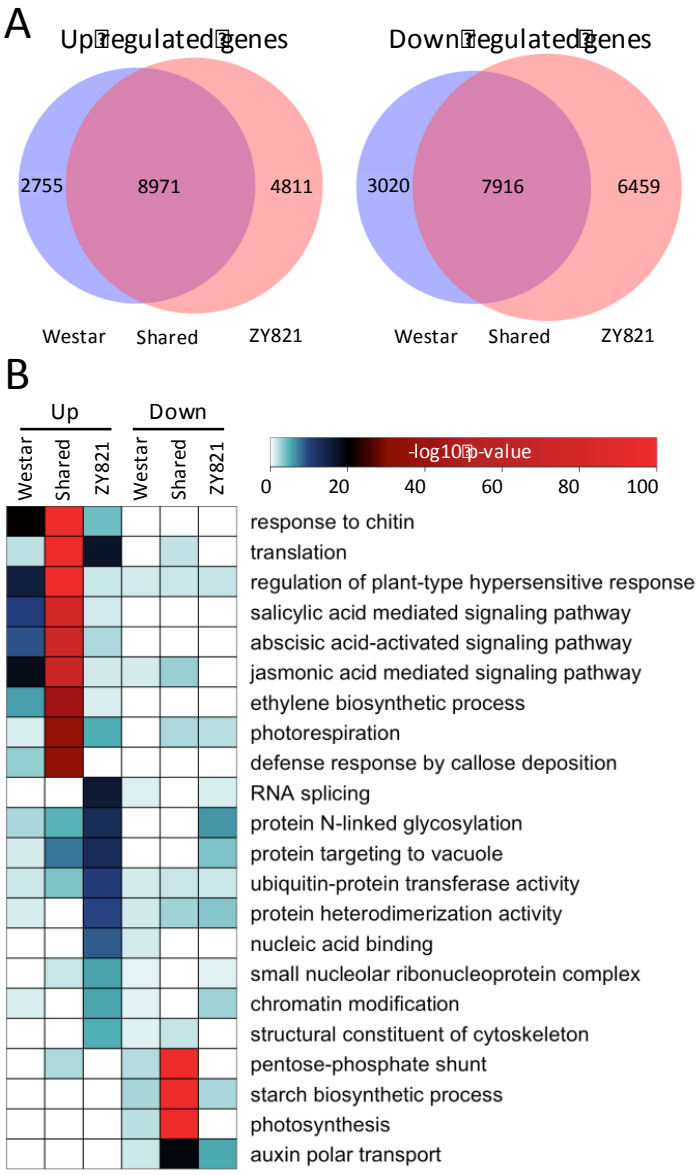

Figure 1.3 Differential gene expression and gene ontology (GO) analysis performed using. (A) Number of genes differentially expressed (false discovery rate < 0.05). (B) Heatmap of a subset of significantly enriched GO terms. GO terms are considered statistically significant if the hypergeometric p-value < 0.001. Dark red and blue colours indicate significance.

Aim 2. To elucidate and identify tissue-specific defense responses to *Sclerotinia* infection that occur in Westar (susceptible) versus Zhongyou821 (tolerant) cultivars.

Research Personnel – Dr. Sanjay Saikia, Research Associate
Mr. Ian Girard, MSc student
Mr. Philip Walker – BSc Co-op student
Ms. Jenna Millar – MSc student

Experimental design and challenges:

- Two canola cultivars viz., Westar (susceptible) and Zhongyou 821 (tolerant)
- Three tissue types from leaves of each cultivar: Epidermis, mesophyll and vasculature
- Treatment duration: 24 hours. Infected and non-infected leaf tissues collected at 24 hours post inoculation with *Sclerotinia*-infected petals
- Leaf tissue processing and microtome sectioning
    - This was one of the biggest challenges experienced to date. While our lab had deep expertise in using this technology on seed tissues, leaves presented a number of challenges. We had to design new protocols for this work. However, we did succeed in the end and have well established and optimized protocols for leaf tissues in canola.
- Laser Capture Microdissection for tissue collection
    - This experiment also presented a new set of challenges. For example, cells of the epidermis are vacuolated and do not contain distinct nuclear or cytoplasmic boundaries compared to cells of the vasculature. Table 1 shows thousands of sections needed for a single biological replicate. This was incredibly time consuming and laborious. We persevered and were successful in collecting enough cells for RNA sequencing.
- RNA isolation from collected tissue followed by RNA sequencing library preparation and sequencing
- Bioinformatics analysis of sequenced data. Similar pipeline presented in Aim 1.
- Identification of novel cell-specific genes and transcriptional regulators
- Functional analysis of specific genes and regulators

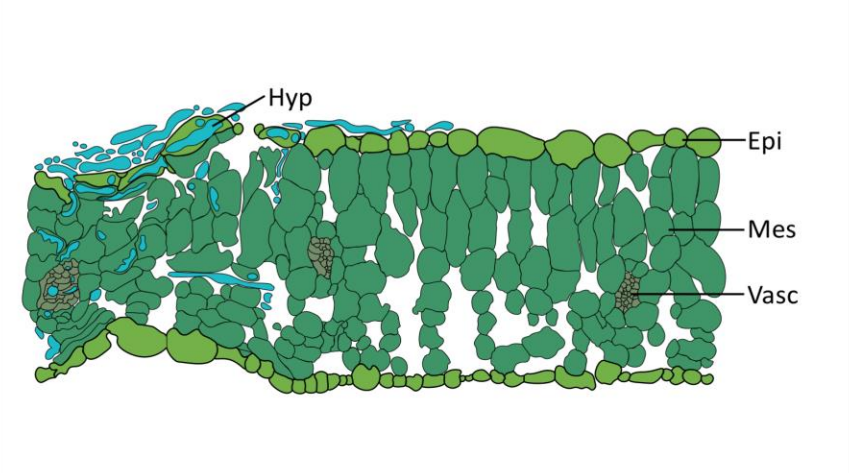

Figure 2.1. Schematic diagram of cells and tissues collected from infected canola leaves. Epidermis (Epi), Mesophyl (Mes), and Vasculature (Vasc) were collected from Sclerotinia- infected (Hyp) and non infected leaves of B. napus 24 hour post inoculation using the petal inoculation method.

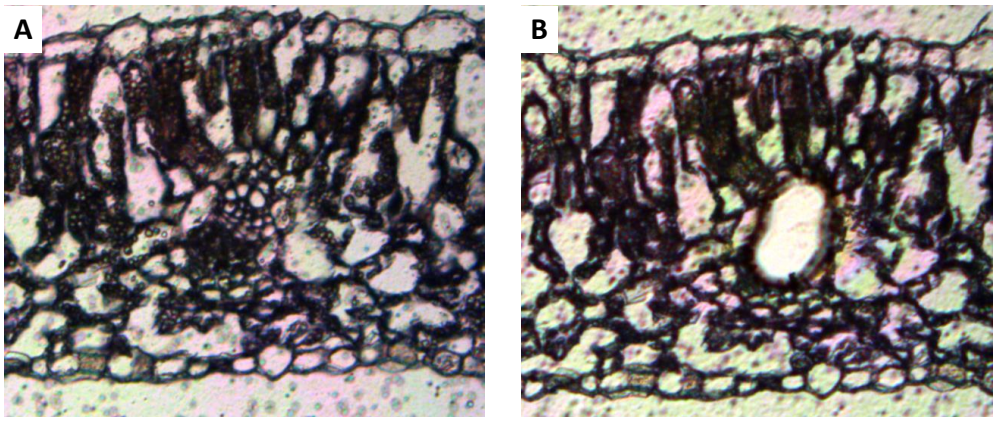

Figure 2.2. Unstained histological sections of susceptible leaf tissues before (A) and after (B) laser microdissection of the vascular bundle. Tissues were sectioned at 10 µm on a Leica ultracut microtome under RNAase free conditions.

Findings:

Table 1. Summary of laser microdissection sample collection, RNA yield and RNA sequence mapped reads to date.

Cultivar-Treatment	Tissue	Biological Replicate	# of LCM elements	RNA yield (ng)	# of mapped reads
Westar-Control	Epidermis	1	1076	4.1	6856766
		2	2556	15.93	TBD
	Mesophyll	1	432	31.8	6225188
		2	402	33.908	TBD
	Vasculature	1	958	14.8	4233909
		2	472	11.1	TBD
Westar-24 hpi	Epidermis	1	2430	10.4	3119461
		2	2551	13.818	TBD
	Mesophyll	1	450	9.5	16445802
		2	550	33	12903262
	Vasculature	1	690	13.2	2283323
		2	580	16.5	8642703
ZhongYou 821-Control	Epidermis	1	2407	18.722	TBD
		2	2800	17.76	TBD
	Mesophyll	1	439	5.7	13550159
		2	400	19.08	TBD
	Vasculature	1	910	10	25952173
		2	899	22.14	TBD
ZhongYou 821-24 hpi	Epidermis	1	1553	8.904	TBD
		2	3897	21	TBD
	Mesophyll	1	450	11.1	10541561
		2	460	14.5	1263829
	Vasculature	1	902	13.886	TBD
		2	900	7.95	TBD

TBD, the samples are currently being sequenced. We expect to have all data analyzed before September 2016.

We collected almost 30,000 histological sections from at least 24 samples of epidermis, mesophyll or vasculature in a susceptible and tolerant cultivar of *B. napus*. To date, we have over 110,000,000 reads to analyze with more on the way.

Bioinformatics analysis of the sequenced data from susceptible leaf cells and tissues challenged with Sclerotinia via the petal inoculation method:

Hierarchical clustering based on expression pattern suggests:

- Hierarchical clustering is used to identify similarities and differences between treatments at the global RNA level
- Tissue lineage is stronger than treatment
- Vascular tissues are transcriptomically distinct compared epidermis and mesophyll. This may be a result of the transport functions of the vasculature compared to the actively photosynthesizing mesophyll or the protective function of the epidermis.

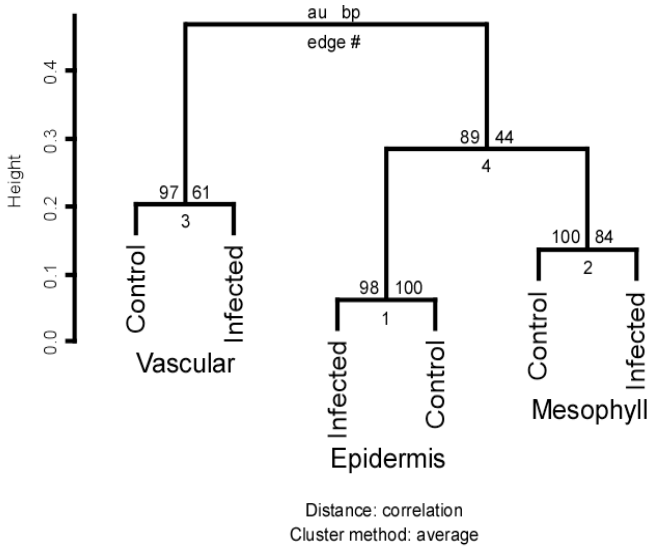

Figure 2.3. Hierarchical clustering of cells and tissues of the susceptible B. napus cultivar, Westar, before and after infection with Sclerotinia using the petal inoculation method on mature leaves.

Distribution of shared and unique genes among the tissue types of susceptible leaves suggests:

- Differentially expressed genes were compared using a Venn Diagram to visualize similarities and differences between gene sets of leaf tissues.
- Gene activity tends to be tissue specific where few genes are shared or overlap between tissues
- Large number of genes are unique to vascular tissue
- Small number of genes shared among tissue types

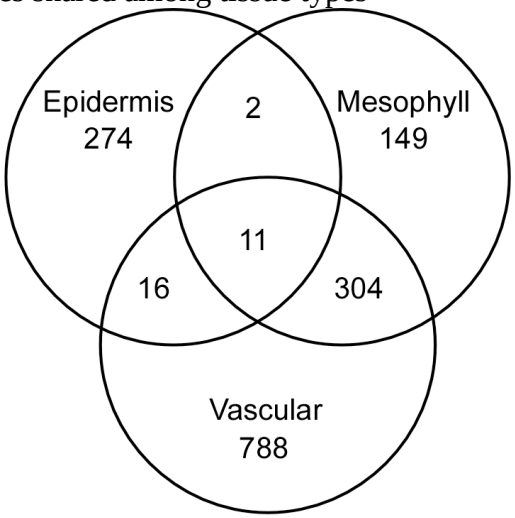

Figure 2.4. Venn diagram showing differences and similarities of differentially expressed genes between the epidermis, mesophyll and vasculature in susceptible leaves of B. napus cv. Westar

Examination of GO terms of infected versus non-infected cells and tissues of susceptible leaves identified enriched biological functions associated with plant defense response including:

- Response to wounding, detection of molecule of fungal origin
- Plant hormone mediated signaling pathways
- Response to chitin, chitinase activity

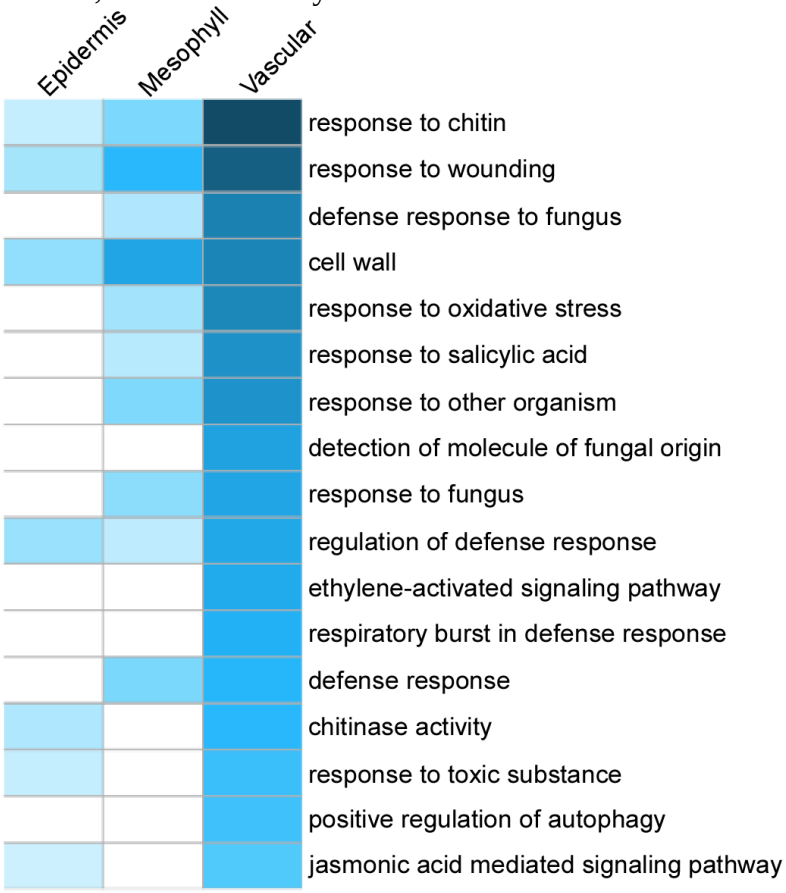

Figure 2.5. Heat map of enriched Gene Ontology terms of differentially expressed genes within the epidermis, mesophyll and vasculature of susceptible B. napus cv. Westar leaves. Darker blue colour represents more significantly enriched biological process

Take home messages:

Sclerotinia infection induces tissue specific defense responses in susceptible leaves of *B. napus* plants.

This data will provide a high resolution, tissue specific atlas of the plant defense response immediately following infection of mature leaves with Sclerotinia and will serve as a valuable resource for researchers interested in developing resistant lines.

On-going studies:

- Sequencing and bioinformatics analysis of the remaining samples listed in Table 1.
- Identification of transcriptional circuits of differentially expressed defense-related gene sets.
- Functional analysis of novel and unique defense-related genes and/or regulators of host pathogen interactions.

Aim 3. To elucidate changes in gene expression and physiology occurring in canola plants in response to the biocontrol bacterium PA23 in the presence and absence of *S. sclerotiorum*.

Research Personnel – Ms. Kelly Duke, MSc student

Experimental Design

- *B. napus* plants were sprayed with solutions of PA23 only, *S. sclerotiorum* ascospores only, or PA23 24 hours prior to *S. sclerotiorum* ascospores and incubated under humid conditions for 72 hours to confirm PA23's efficacy at preventing fungal infection *in planta*.
- Leaf tissue from these plants was collected and used for follow-up experiments, including: RNA isolation and cDNA library synthesis for RNA sequencing (RNA-seq), tissue staining for reactive oxygen species (ROS), and transmission electron microscopy (TEM).

Key Findings

1) **PA23 reduces *S. sclerotiorum* infection rates in *Brassica napus*.** When comparing the rate of infection as the proportion of lesion-forming petals to total petals fallen into the plant canopy, application of PA23 reduced the number of lesions present on plant leaves by more than 91% (Fig. 1A). Using this infection model, leaf necrosis was visible under lesion-forming petals as soon as 24 hours post infection with *S. sclerotiorum* in plants receiving pathogen treatment only (Fig 1B).

2) **RNA-seq reveals global patterns of gene expression.** A total of 48,454 genes with Fragments Per Kilobase of transcript per Million mapped reads (FPKM) ≥ 1 were detected across all samples, representing 48% of the predicted canola gene models (Fig 2).
3) **Gene ontology enrichment analysis reveals biological processes in *B. napus* activated by treatment with PA23 and/or *S. sclerotiorum*.** RNA-seq data generated lists of shared and unique differentially expressed genes (DEGs) between treatments over the water control, and these lists were used for gene ontology enrichment analysis (Fig.3 A,B). Plants treated with *S. sclerotiorum* alone had the greatest number of uniquely upregulated DEGs at 8,237 genes. Plants treated with both PA23 and *S. sclerotiorum* had the fewest unique upregulated DEGs at 515 genes, a 16-fold reduction compared to the *S. sclerotiorum* treatment group. All treatment groups were enriched for response to reactive oxygen species (ROS) and systemic acquired resistance (SAR) over the water control. Several GO terms associated with the chloroplast were enriched in response to PA23, including thylakoid, plastid translation and plastoglobule enrichment in the PA23 only treatment group, and plastid thylakoid membrane as well as negative regulation of chlorophyll catabolic process enrichment in both PA23 only and PA23+Ss treatment groups. As a result, these three aspects (ROS, SAR, chloroplast activity) were further investigated.
4) **PA23 prevents ROS accumulation in the leaf.** One of the first responses to environmental stressors in plants is the production of reactive oxygen species (ROS). ROS can function both as a direct immune response to invading pathogens and also as signaling molecules for the onset of a systemic response. Figure 4 depicts ROS production among treatment groups using staining to reveal hydrogen peroxide and superoxide radicals. Whereas *S. sclerotiorum*-treated samples were inundated with both hydrogen peroxide and superoxide radicals in the regions directly surrounding lesions (Fig. 4M-P), ROS production was greatly reduced when plants were treated with PA23 ahead of the fungal pathogen (Fig. 4I-L). PA23-treated leaves had no regions containing large aggregations of these molecules, although hydrogen peroxide production appeared similar to the PA23+Ss treatment group (Fig. G, H).
5) **PA23 treatment results in multiple structural and metabolic changes in the *B. napus* chloroplast.** As chloroplasts are organelles central to important cellular processes such as photosynthesis and the synthesis of various short-and long-range signaling molecules, we were interested to see what structural changes were occurring in response to combinations of PA23 and *S. sclerotiorum*. Transmission electron microscopy revealed that when plants were treated with PA23, the area dedicated to thylakoid structures showed an increase in granal stack organization and the accumulation of plastoglobuli within 24 and 48 hours post inoculation (Fig. 5). While gene expression in plants treated with PA23 in combination with *S. sclerotiorum* indicated significant upregulation of starch metabolic processes (Fig. 3B), these chloroplasts were similar in appearance to those of the biocontrol only-treated group with many grana stacks and plastoglobules (Fig. 5).
6) **SAR is activated in all treatment groups but involves different sets of genes and expression patterns.** Systemic defense responses like systemic acquired resistance (SAR) allow the host plant to develop an enhanced resistance to future pathogen attack even in organs that have not previously been exposed to a pathogen. In order to

understand what, if any, systemic responses were occurring in *B. napus* in response to PA23, we examined gene expression changes in key genes associated with SAR. Figure 6 contains SAR-related genes which are differentially expressed among treatment groups. Three broad categories of expression were observed: (1) genes which were upregulated when *S. sclerotiorum* was present only, (2) genes upregulated or downregulated across all three treatment groups, and (3) genes upregulated in PA23 only group but downregulated when *S. sclerotiorum* was present. Category (3) indicates unique SAR-related pathways being induced in response to PA23.

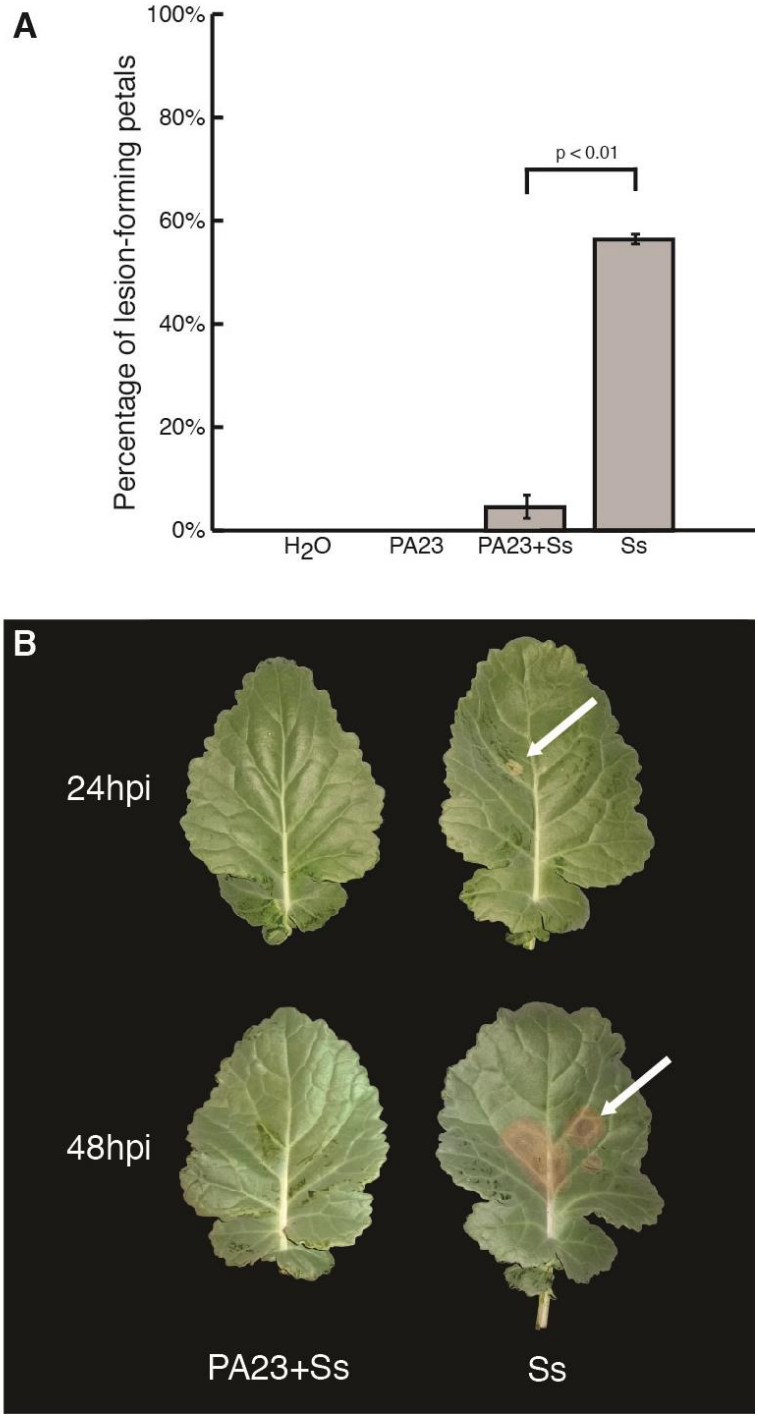

***Figure 3.1**. Reduction of S. sclerotiorum infection rates by P. chlororaphis PA23. A. Numbers of lesion-forming petals as a percentage of total petals which fell onto plant leaves in greenhouse assays. B. S. sclerotiorum disease progression on canola leaves at 24 hours or 48 hours after petal application. PA23+Ss treatment petals were inoculated with PA23 24 hours prior to S. sclerotiorum inoculation, whereas Ss treatment petals were inoculated with sterile water. Both treatment groups (PA23+Ss and Ss) petals were then infected in vitro with S. sclerotiorum 48 hours prior to being placed on leaves.*

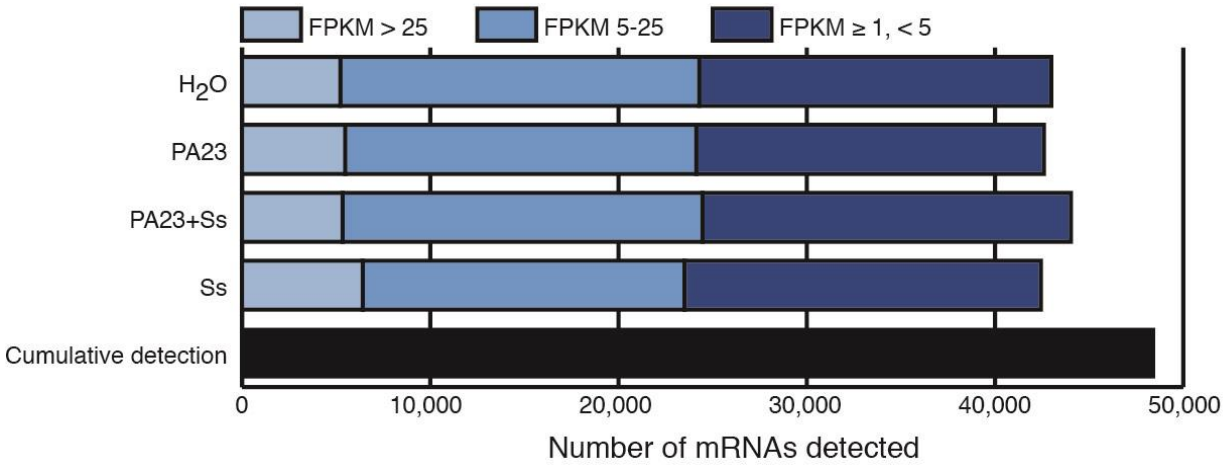

Figure 3.2. *Number of unique mRNAs present in treatment groups, as well as cumulative number of unique mRNA transcripts identified. Transcripts are categorized by frequency of occurrence in the library, as described by the number of fragments per kilobase of transcript per million mapped reads (FPKM) value.*

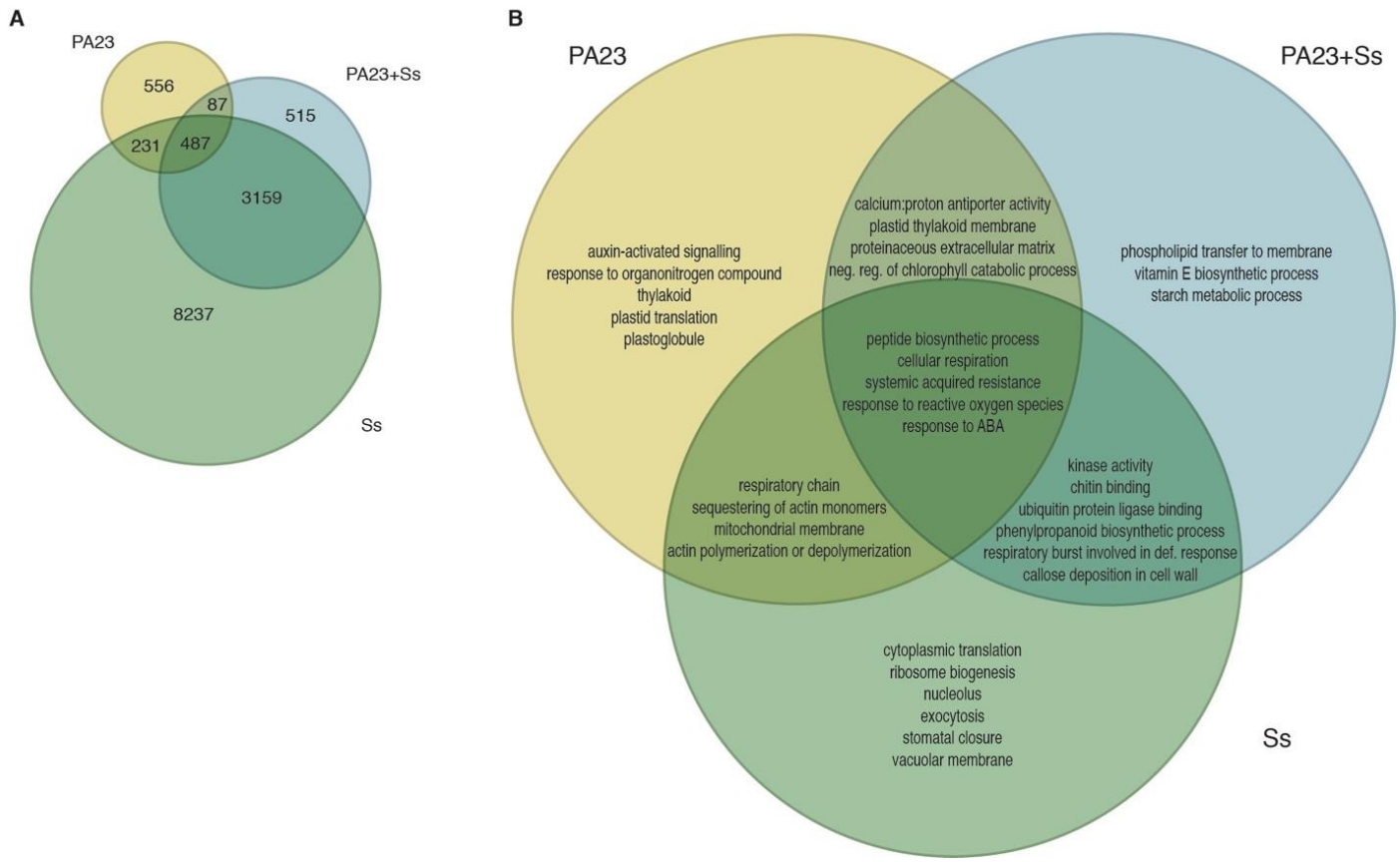

***Figure 3.3**. Gene expression changes unique to bacterial or fungal treatments of canola leaves. A. Venn diagram of B. napus gene counts for uniquely and significantly upregulated genes in treatment groups compared to the water control. B. Venn diagram of enriched GO terms selected from upregulated genes in A.*

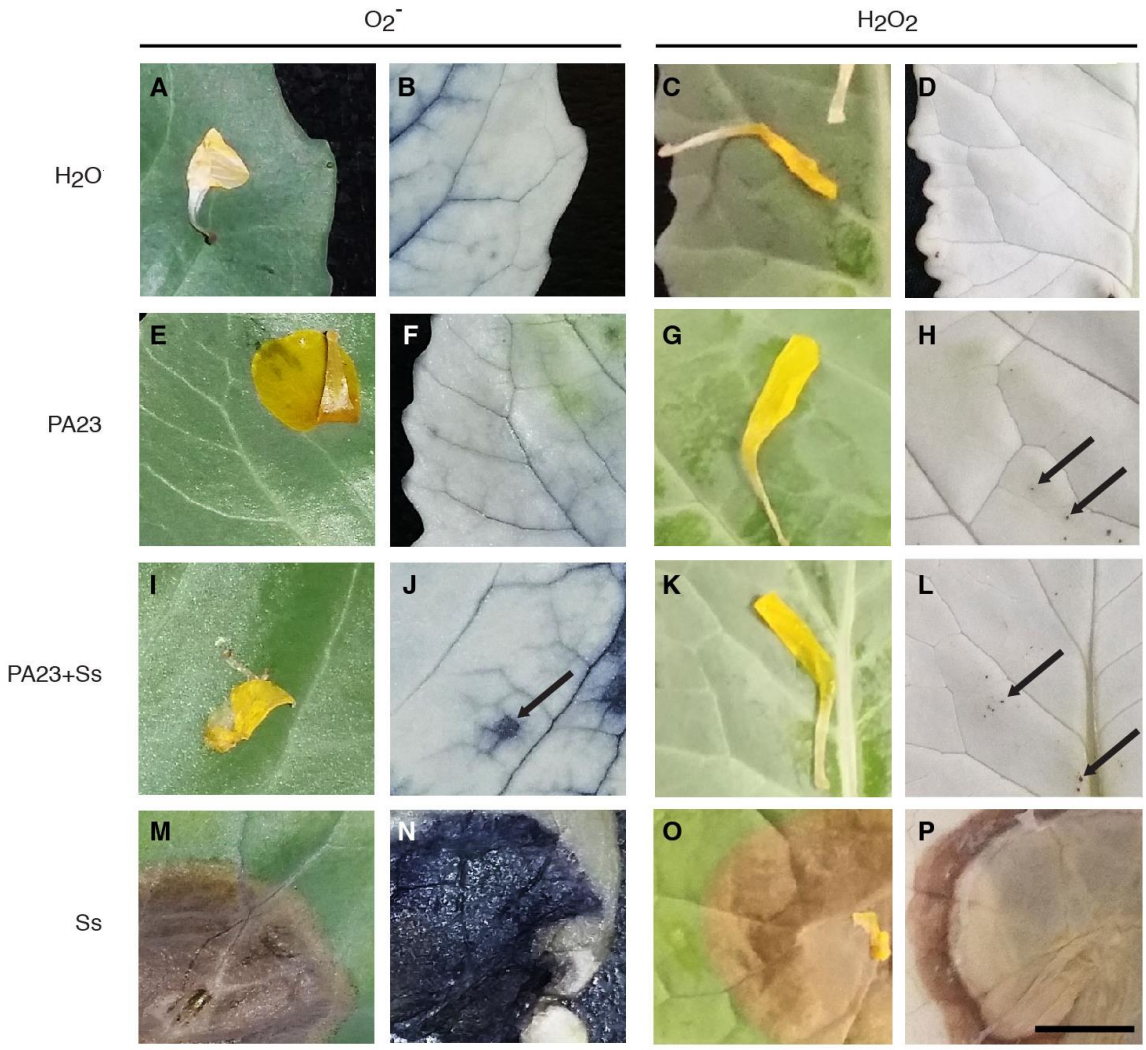

Figure 3.4. *Detection of superoxide radicals (left) and hydrogen peroxide (right) in canola treatment groups. The leftmost column in each set depicts leaves after treatment and before staining. The rightmost column depicts the same area of tissue after petal removal, staining and treatment to remove leaf pigmentation. Scale bar in (P) = 5mm and is applicable to panels (A) - (P).*

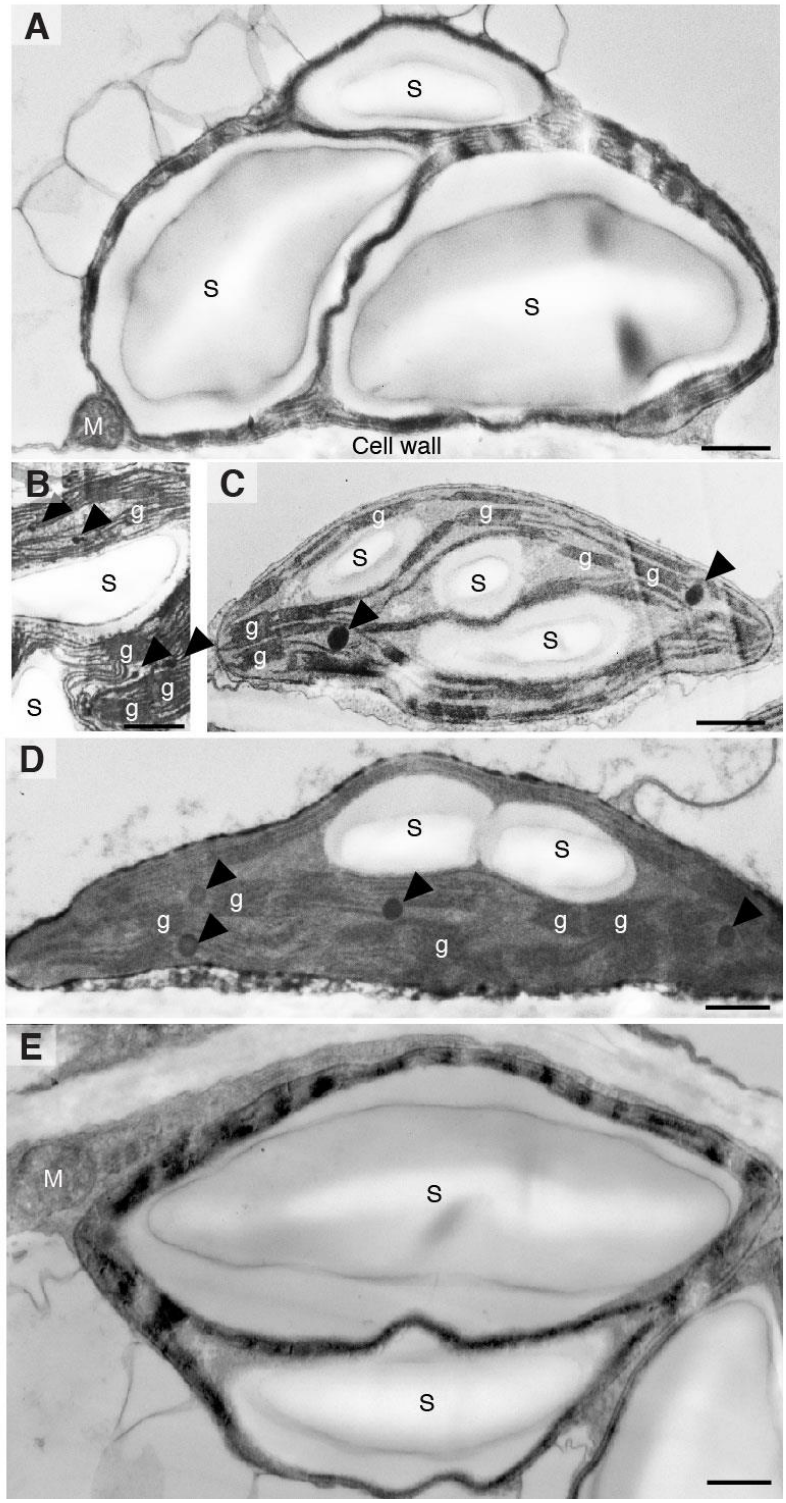

Figure 3.5. *Transmission electron micrographs of leaf chloroplasts. A. Water control, 48hrs. B. PA23, 24 hrs. C. PA23, 48 hrs. D. PA23+Ss, 24 hrs. E. Ss, 48 hrs. S=starch granule; g=grana stack; M=mitochondria. Arrows indicate plastoglobules. Scale bar for panels A-E = 500nm.*

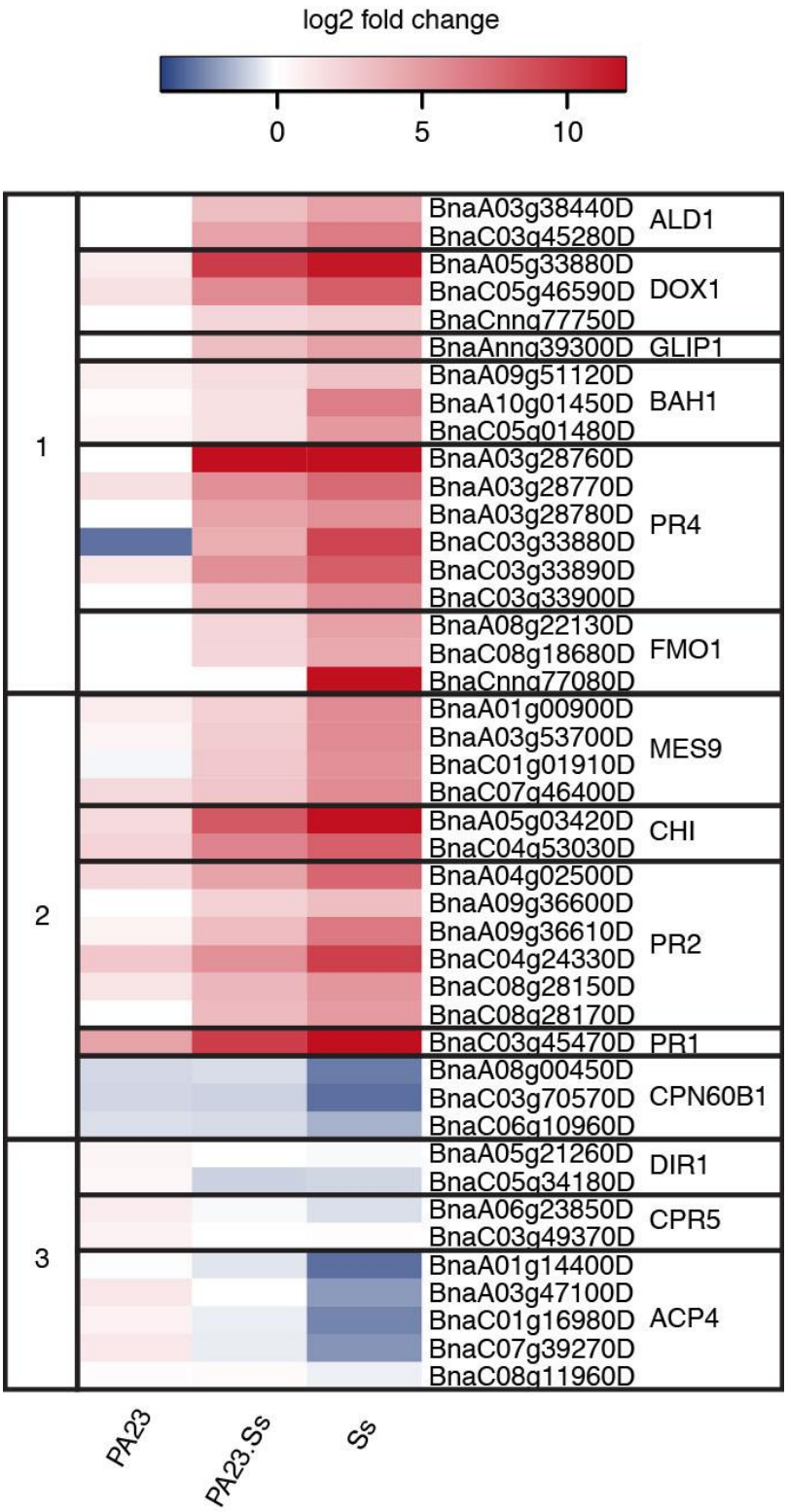

Figure 3.6. *Comparison of transcript abundance of select SAR-associated genes as determined by RNA-seq. Transcript abundance is measured in log2 fold change. The leftmost column groups genes by expression pattern.*
Conferences and presentations

Take Home Messages:

Taken together, data reveal PA23 has a significant effect on the host plant in the *B. napus*-*S. sclerotiorum* pathosystem. PA23 likely operates through modulation of defense responses in the plant and through the induction and activation of unique gene expression patterns directly at the host pathogen interface. This has relevance for the use of PA23 as a biocontrol agent in the field and in commercial applications, as we have shown that PA23 application functions not only via antibiosis of the pathogenic fungus *S. sclerotiorum*, but also via activation of unique responses in the chloroplast and related to SAR. Further investigation into these processes could be accomplished through time course experiments, comparisons of responses using PA23 knockout mutants, or a comparison of plant response to phyllosphere application of PA23 versus soil applications such as root dips or irrigation.

Dissemination of Research Findings:

Papers

Girard IJ, Mcloughlin AG, de Kievit T, Fernando WGD, Belmonte MFB. Integrating large-scale data and RNA technology to protect crops from fungal pathogens. *Frontiers in Plant Science.* Accepted and in press (2016).

Selin C, de Kievit TR, Belmonte MF, and Fernando WGD Elucidating the role of effectors in plant-fungal interactions: progress and challenges. *Frontiers in Plant Science* Accepted and in press (2016).

*Girard IJ, *Tong C, Mao X, Becker MG, de Kievit T, Fernando WDG, Li G, Belmonte MF. Global RNA profiling of the initial *Sclerotinia sclerotiorum – Brassica napus* infection process reveals cross talk between redox, hormone and carbon metabolism pathways. To be submitted to Molecular Plant, May 2016. *Denotes co-first authorship

Duke K, Belmonte MF, Fernando D, and de Kievit, TR. Defense patterns induced in *Brassica napus* in response to the biocontrol agent *Pseudomonas chlororaphis* PA23. To be submitted to Plant Journal, June 2016.

Conferences and presentations

Girard IJ, de Kievit T, Fernando WDG, Belmonte M. (2015) Using big data to probe the plant defense response: RNA sequencing of the canola sclerotinia pathosystem. **Oral presentation**, December 7th 2015. Regional Canadian Phytopathological Society Meeting: Manitoba Chapter. Awarded Honourable Mention for the presentation competition.

Girard IJ, de Kievit T, Fernando WDG, Belmonte M. (2015) Using big data to probe the plant defense response: RNA sequencing of the canola sclerotinia pathosystem. **Oral presentation**, 2015. Botany 2015: Science and plants for people. Edmonton, MB, Canada.
Awarded Honourable Mention for Canadian Society of Plant Biologists Student Competition

Girard IJ, Belmonte MF. (2015) Using big data to probe the plant defense response: RNA sequencing of the canola sclerotinia pathosystem. **Oral presentation**, 2015 Prairie University Biology Symposium, Winnipeg, MB, Canada.

Girard IJ, Fernando D, Becker MG, Belmonte MF. (2014) Bull's-eye: tissue processing improvements for isolating high quality RNA from laser microdissected pathogen infected cells and tissues. **Technical session (oral) presentation**, 2014 American Phytophathological Society – Canadian Phytopathological Society Joint Meeting, Minneapolis, MN, USA.

Duke K, Belmonte MF, Fernando D, and de Kievit, TR. (2015). Primed defense patterns in canola in response to the biocontrol agent *Pseudomonas chlororaphis* PA23. **Poster Abstract 60**. ASM International Conference on *Pseudomonas*, Washington DC, Sept 8 -12, 2015.

Duke K, Belmonte MF, Fernando D, and de Kievit, TR. (2016). The regulation of defense responses in canola in response to the biocontrol agent *Pseudomonas chlororaphis* PA23 revealed by RNA-seq. **Poster Abstract.** The Canadian Biochemical Society, Winnipeg, MB, June 1-3, 2016.