**Final Project Report to SCDC**

**Date:** March 31, 2010

**Reporting Period:** October 2007 to March 2010

**Investigators:** Dwayne Hegedus, Roger Rimmer and Lone Buchwaldt

**Project Title:** A Genomics Approach to Sclerotinia Resistance in *Brassica napus*

## Executive Summary

The SCDC project was one of several research projects undertaken between 2005 and 2009 to develop resources and information to address sclerotinia resistance in canola. These were funded by Saskatchewan Agriculture and Food, the Saskatchewan Canola Development Commission (SCDC), a consortium of canola breeding companies and by the AAFC Matching Investments Initiative (MII). We have been successful in continuing this cost-sharing arrangement to link resources from federal and provincial governments, producers and the canola breeding industry to generate the genetic information and breeding tools needed for successful development of sclerotinia resistant cultivars. To date, Saskatchewan Agriculture and Food has agreed to continue funding through to 2013. SCDC has also agreed to continue its support of this work for the same period and and an application to the Developing Innovative Agri-Products (DIAP) initiative to provide federal funding was approved. Discussions with the industry partners are under way to renew the consortium.

The long term objective of the SCDC project is to provide the canola industry with specific resistance genes and molecular markers for developing stem rot resistant *Brassica napus* cultivars. In this project our objectives were:

1) To identify candidate resistance genes using genomics methods, such as expressed sequence tags (ESTs) and gene arrays.
2) To examine the contribution of select genes to resistance.
3) To develop a better understanding of the factors employed by the fungus to cause disease and to determine how the plant inactivates these factors.

The project received a one-year extension (2009-2010) which allowed a summer student and a casual technician to be hired to complete project objectives with originally allocated funds.

Markers are essential to introduce resistance genes into suitable canola breeding lines. Resistance genes identified using genomics approaches have contributed to the development of these markers, and vice versa. The winter-type *B. napus* Zhong You 821 (ZY821) developed in China is one of the best cultivars for resistance to stem rot. At AAFC, we employed genomics approaches to identify the genes responsible for sclerotinia resistance in ZY821. Using powerful techniques, such as cDNA subtraction and DNA micro-arrays, we identified the suite of genes expressed in ZY821 in response to *S. sclerotiorum* infection. In a parallel project, doubled haploid (DH) lines from several resistant ZY821 x susceptible *B. napus* crosses (mapping populations) were developed, and we used this material to identify genes expressed specifically in resistant but not in susceptible lines. Several putative resistance genes were introduced into a susceptible *B. napus* line to determine their individual contribution to resistance. Several other genes identified in these experiments were mapped to resistance loci which will facilitate introgression of the resistance genes into elite canola lines.

The second component of the project examined how *S. sclerotiorum* causes disease and the mechanisms used by the plant to prevent this. In this regard, five polygalacturonases (enzymes that break down pectin) and two necrosis-inducing proteins were characterized and found to be the principle factors responsible for the development of necrotic lesions. Using a genomics approach, the genes encoding 17 *B. napus* polygalacturonase inhibitor proteins were identified and characterized. Two of these was found to inhibit the activity of *S. sclerotiorum* polygacturonases and are being evaluated as potential resistance gene candidates.

**Technical Report:**

In cases where the work arising from the project was published, the technical report provides only a summary of the most important findings. Additional detail is provided in the publications and appendices associated with this document which are listed at the end of the appropriate sections.

**Objective I: Identification of genes associated with resistance in *B. napus* ZY821**
**Subproject A: Examining Gene Expression Patterns**

I. Patterns of gene expression in susceptible and resistant *B. napus* lines

To simulate natural infection of *B. napus* stems by *S. sclerotiorum* under field conditions, we attached mycelial plugs to the stems of flowering plants. Visible lesions developed within 24 - 48 hours post inoculation (hpi). After 7 days, lesions on stems of the resistant cultivar ZY821 were smaller than on the susceptible cultivar Westar, and most Westar plants died within 21 days of inoculation (Figure 1).

Identifying the induced defense genes was an important first step toward understanding sclerotinia resistance. Initially, we developed a subtractive cDNA library from infected vs non-infected stems of the resistant *B. napus* line ZY821 and identified 76 genes that were expressed in response to *S. sclerotiorum* inoculation. A subset of these genes was found to be highly induced in response to *S. sclerotiorum* inoculation, including those encoding anti-fungal proteins such as the pathogenesis-related proteins (PR) PR1 and PR5, chitinase (5 types), endoglucanase and two lectins (Figure 2). However, it was equally likely that resistance was due to alterations in the timing of induction (i.e. earlier) and/or to higher levels of expression of these defenses, which would be determined by a small number of regulatory proteins. Members of gene families, such as regulatory proteins, are generally under-represented in subtractive cDNA libraries since they share a high degree of DNA sequence similarity. To overcome this problem, we used a *B. napus* oligonucleotide microarray representing 15,000 unique genes to study the response in stems to inoculation with *S. sclerotiorum* in resistant and susceptible cultivars. The

micro-array was designed by AAFC and was the most comprehensive *B. napus* array available at that time.

A large number of genes were found to be differentially regulated after infection (Table 1; Figure 3; Figure 4). The gene expression profiles during the latter stages of stem infection were similar to that of *B. napus* seedlings in response to *S. sclerotiorum* infection (Yang *et al.*, 2007; Zhao *et al.*, 2007) and other necrotrophic pathogen-host interactions, namely *Botrytis cinerea* - *A. thaliana* and *Alternaria brassicicola* - *Brassica oleracea*. We biased our sampling toward the very early stages of the infection since events occurring soon after the initial interaction between pathogen and host are likely to be critical for resistance to aggressive necrotrophs such as *S. sclerotiorum*. We chose to examine infected stems since this represented the natural site for infection in this pathosystem. Indeed, we observed that the resistant line, ZY821, induced defense-associated genes sooner than the susceptible line, Westar (Table 2) as well as a unique set of genes (Table 3). We also observed differences in the expression patterns of a large group of regulatory genes, as well as genes involved in plant hormone synthesis and aspects of the host defense mechanisms against pathogens (Figure 5). Changes in the expression of genes involved in carbon metabolism suggest that carbon storage reserves (such as sucrose, starch and lipid) are accessed and shuttled through the photorespiration pathway. This pathway leads to the formation of glyoxylate that can enter the TCA cycle, as well as hydrogen peroxide that may form a part of the defense response. The activity of the TCA cycle may be greatly increased as evidenced by the induction of many genes encoding TCA cycle enzymes. This not only generates reducing power and energy, but also precursors for amino acid biosynthesis (Figure 6). We observed a sharp increase in transcript abundance for genes encoding enzymes for tryptophan biosynthesis, this amino acid being a precursor for both glucosinolates and phenylpropanoids (Figure 7). This study has provided new insight into aspects of the defense response to necrotrophic pathogens using an experimental design that closely models the actual circumstances occurring in the field. We are currently exploring several targets to determine their contribution to resistance in *B. napus* ZY821 and other resistant cultivars.

See the following appendices for additional details:

Zhao J., Buchwaldt, L., Rimmer R., Sharpe A., Bekkaoui D, Hegedus DD. (2009) Patterns of differential gene expression in *Brassica napus* cultivars infected with *Sclerotinia sclerotirum*. Mol. Plant Pathol. 10: 635-649.

Files on disk:

Supplementary Table S1. List of differentially expressed genes in *B. napus* cv. Westar

Supplementary Table S2. List of differentially expressed genes in *B. napus* cv. ZY821

Supplementary Table S3. Comparison of inoculated *B. napus* cv. ZY821 and Westar

II. Effect of polyploidization on the expression and function of *B. napus* defense genes

Gene redundancy due to polyploidization provides a selective advantage for plant adaptation. In the context of the response to the fungal pathogen *S. sclerotiorum*, we examined the expression patterns of two peroxidase genes (*BnPOX1* and *BnPOX2*) in the natural allotetraploid *B. napus* and the model diploid progenitors *Brassica rapa* (Br) and *Brassica oleracea* (Bo). We demonstrated the Bo homeolog of *BnPOX1* was up-regulated after infection, while both *BnPOX2* homeologs were down-regulated. A bias toward reciprocal expression of the homeologs of *BnPOX1* in different organs in the natural allotetraploid of *B. napus* was also observed. These results suggest that subfunctionalization of the duplicated *BnPOX* genes after *B. napus* polyploidization, as well as subneofunctionalization of the homeologs in response to this specific biotic stress has occurred. Retention of expression patterns in the diploid progenitors and the natural allotetraploid in some organs indicates that the function of peroxidase genes was conserved during evolution. This study is important as it is now apparent that the function of some genes in diploid progenitors may not be retained in the polyploid derivatives. This will have to be taken into consideration when evaluating the introgression of genes from wide crosses and resynthesized polyploid lines.

See the following appendices for additional details:

Zhao, J., Buchwaldt, L., Rimmer, S.R., Brkic, M., Bekkaoui, D. and Hegedus, D.D. (2009) Differential expression of duplicated peroxidase genes in the allotetraploid *Brassica napus*. Plant Physiol. Biochem. 47: 653-656.

**Subproject B: Relationship between ESTs and Quantitative Trait Loci (QTL) for Resistance**

In an allied project (investigators: Buchwaldt, Rimmer and Lydiate) quantitative trait loci (QTL) conferring resistance to sclerotinia stem rot in ZY821 are being mapped. Several populations of doubled haploid lines and recombinant inbred lines were developed from crosses with the resistant line into both spring type and winter type back grounds. The progenies were genotyped with microsatellite markers and phenotyped by inoculation of stems with *S. sclerotiorum* (isolate #321). QTL analysis by composite interval mapping (QTL Cartographer) of the combined data set has identified six QTL in spring type populations and four in winter type populations. The most significant QTLs mapped to linkage groups N5, N6, N7, N16 and N19 with LOD values between 7 and 10 and phenotypic variability explained between 19 and 43 %. Microsatellite markers flanking the QTL were identified and fine mapping of the QTL intervals is continuing.

The location of genes that were induced in response to *S. sclerotiorum* infection was mapped relative to the QTL using the high degree of synteny with the *A. thaliana* genome. Dr. Isobel Parkin has conducted the comparative genomic analysis. Cleaved amplified polymorphic sequence (CAPS) markers rely on minor DNA sequence variations that alter restriction enzyme sites in the amplified products from different alleles. Gene sequences in the AAFC Brassica EST database and homologous *A. thaliana* sequences are being used to develop PCR primers to amplify the corresponding DNA from the parental lines of the DH populations. Amplification products will be cut with different restriction enzymes and run on agarose gels to identify the restriction enzyme that provides polymorphism between the two parental lines. We expect some CAPS markers will map to the known QTL intervals while others will represent new resistance loci. Some candidate genes from ZY821 are currently being developed as single nucleotide polymorphism (SNP) markers in another project at AAFC. As these markers become available they will be used to supplement the CAPS markers for mapping candidate genes.

**Subproject C: Selection and testing candidate genes for resistance to stem rot**

I. *B. napus* Polygalacturonase Inhibitory Proteins (PGIP)

Plants encode a distinct set of PGIP that function to inhibit polygalacturonase (PG) enzymes produced by soft rot fungal pathogens. Characterization of *S. sclerotiorum* PGs is described in the report for Objective II below. Initially, we characterized two PGIP-encoding genes (*Bnpgip1* and *Bnpgip2*) from *B. napus* DH12075 (a doubled haploid line derived from a cross between 'Crésor' and 'Westar'). The two proteins exhibited 67.4% identity at the amino acid level and contained 10 imperfect leucine-rich repeats. The *pgip* genes appeared to be members of a small multigene family in *B. napus* with up to 4 members. *Bnpgip1* and *Bnpgip2* were constitutively expressed in root, stem, flower bud and open flowers. In mature leaf tissue, different levels of induction were observed in response to biotic and abiotic stresses. *Bnpgip1* expression was highly responsive to flea beetle feeding and mechanical wounding, weakly to *S. sclerotiorum* infection and exposure to cold but not to dehydration. Conversely, *Bnpgip2* expression was strongly induced by *S. sclerotiorum* infection and to a lesser degree by wounding but not by flea beetle feeding. Application of jasmonic acid to leaves induced both *Bnpgip1* and *Bnpgip2* gene expression; however, salicylic acid did not activate either gene. Taken together, these results suggested that separate pathways regulate *Bnpgip1* and *Bnpgip2* and their role in plant development or resistance to biotic and abiotic stress differs. The two cDNA fragments were used separately to screen a *B. napus* DH12075 bacterial artificial chromosome (BAC) library. BAC clones with unique restriction digest patterns were sequenced and a set of at least 17 PGIP genes similar to *Bnpgip1* or *Bnpgip2* were discovered. This is the largest *pgip* gene family reported to date. Comparison of the BnPGIPs revealed several sites within the xxLxLxx region of leucine rich repeats that form β-sheets along the interacting face of the PGIP that were hypervariable and represent good candidates for generating PGIP diversity. Characterization of the regulatory regions and RT-PCR studies with gene-specific primers revealed that individual genes were differentially responsive to pathogen infection, mechanical wounding and signaling molecules. Many of the *Bnpgip* genes responded to infection by the necrotic pathogen, *S. sclerotiorum*; however, these genes were also induced either by jasmonic acid, wounding and salicylic acid or some combination thereof. The large number of PGIPs and the differential

manner in which they are regulated likely indicates that *B. napus* has an inherent ability to respond to attack from a broad spectrum of pathogens and pests.

II. Interaction of *S. sclerotiorum* polygalacturonases with *B. napus* PGIP

A graduate student was employed to determine which of BnPGIPs inhibit the activity of *S. sclerotiorum* polygalacturonases (SSPG). The secretory yeast *Pichia pastoris* was transformed with the vector, pPICZa, containing cassettes designed to express and secrete hexahistidine-tagged SsPG1, SsPG2, SsPG3, SsPG5, SsPG6 as well as BnPGIP1, BnPGIP2, BnPGIP7 and BnPGIP16 in a methanol inducible manner. PGIP1 was expressed at the highest level of all the PGIPs. PGIP7 and PGIP16 were expressed at somewhat lower levels and PGIP2 expression was undetectable in either of lines.

Of the five SSPGs examined, SSPG3 and SSPG6 were expressed at very high levels. The level of SSPG5 was not sufficient to be detected by staining with Coomassie blue after SDS-PAGE separation but was detectable by western blot analysis. These SSPGs were concentrated successfully using Centricon tubes. SSPG1 expression was not detectable by western blot analysis of neat culture supernatant, but was detectable after concentration of the samples. The SSPGs were shown to possess pectinolytic activity.

In order to characterize the biochemical requirements for the individual PGs and to examine their interaction with PGIPs it was necessary to develop a consistent assay to quantify PG activity. Dinitrosalisylic acid (DNSA) interacts with free galacturonic acid (GA) released by PG activity on pectin to form a colored compound that can be quantified using a spectrophotometer. Several modifications of the existing protocol were required to stabilize the readings and make the assay more robust. PG-PGIP interaction studies were conducted with the concentrated PGIPs and SSPG3 and SSPG6. PGIP1 and PGIP7 were found to inhibit SSPG6 and PGIP16 was found to consistently inhibit the activity of SSPG3 (Figure 8).

III. Testing of Candidate Resistance Genes in *B. napus* and *A. thaliana*

We have begun evaluating candidate resistance genes by expressing them in the susceptible *B. napus* cultivar DH12075 and the model plant *A. thaliana*. Homozygous transformed lines were

identified and were/are being rated for disease resistance (Table X). The initial set of candidate genes was selected from the earlier subtractive cDNA library and included a two hevein-type lectins and two proteins (BnPGIP1 and BnPGIP2) that inhibit the activity of pathogen enzymes needed to destroy plant tissues. The constructs were designed to over-express the genes constitutively (present in all tissues all the time) or to express them only in the stem in response to sclerotinia infection (using an inducible promoter from one of the *Bnpgip* genes above).

A second set of candidate genes was derived from the micro-array experiments comparing resistant and susceptible lines in subproject A. One of these was a WRKY-type transcription factor that has been implicated in inducing defense response in other plants. Genes encoding three WRKY factors, BnWRKY33, BnWKY40 and BnWKY53 (named according to the most closely related *A. thaliana* ortholog), were induced within 6-12 hpi in ZY821. As these may be important for regulating the expression of genes encoding defense proteins, we examined the signaling pathways required for induction of *BnWRKY33* gene expression since it was associated with a QTL marker for *S. sclerotiorum* resistance in *B. napus* (Zhao and Osborn, 2007). Two other candidate genes were over-expressed in *B. napus*. These encode a concanavalin-like lectin, the expression of this gene was up-regulated 20,000 fold after *S. sclerotiorum* infection, and annexin, a protein that is involved in cytoskeleton organization and believed be involved in the delivery of defense proteins to the site of infection. Homozygous, single insert lines have been generated and being evaluated for disease resistance.

Oxalate oxidase breaks down the oxalic acid released by the pathogen and has been shown to provide resistance to *S. sclerotiorum* in soybean (D. Simmonds, pers. comm.). We evaluated *B. napus* lines expressing wheat oxalate oxidase and did not observe an increase in resistance. Therefore, we generated *A. thaliana* lines expressing a wheat oxalate oxidase gene, either alone or in combination with *BnPgip1* or *BnPgip2*. This material is currently being evaluated.

See the following appendices for additional details:

Hegedus DD, Li, R., Buchwaldt L., Parkin I., Whitwill S., Coutu C., Bekkaoui D, Rimmer SR. (2008). *Brassica napus* possesses an expanded set of polygalacturonase inhibitor protein

genes that are differentially-regulated in response to *Sclerotinia sclerotiorum* infection, wounding and defence hormone treatment. Planta 228:241-253.

Li, R., Rimmer, R., Yu, M., Sharpe, A.G., Séguin-Swartz, G., Lydiate, D. and Hegedus, D.D. (2003) Two polygalacturonase inhibitory protein genes are differentially expressed in response to biotic and abiotic stresses in *Brassica napus*. Planta 217: 299-308.

**Objective II: *S. sclerotiorum* genes associated with pathogenesis**

I. Genes expressed during *S. sclerotiorum* gene infection.

Initially, we identified *S. sclerotiorum* genes expressed during infection (or a condition that simulated infection) through EST analysis (Li et al. 2004a, b). To identify genes involved in fungal pathogenesis we generated 2232 ESTs from two cDNA libraries constructed using either mycelia grown in pectin medium or tissues from infected *B. napus* stems. A total of 774 individual fungal genes were identified of which 39 were represented only among the infected plant EST collection. Annotation of 534 unigenes was possible following the categories applied to *Saccharomyces cerevisiae* and the Universal Gene Ontology scheme. cDNAs were identified that encoded potential pathogenicity factors including four endopolygalacturonases, two exopolygalacturonases and several metabolite transporters. A non-redundant set of *S. sclerotiorum* ESTs was deposited in Genbank (Accession CD645592-CD646371, CF542256-CF542258, CF602382-CF602389) and COGEME (cogeme.ex.ac.uk) databases.

II. Characterization of *S. sclerotiorum* enzymes involved in *B. napus* infection

The main pathogenicity determinants expressed by *S. sclerotiorum* are oxalic acid and the enzyme polygalacturonase (PG). Oxalic acid is toxic to host tissue as it acidifies the environment and binds calcium in the plant cell wall leading to loss of cell wall integrity (Godoy, 1990). Polygalacturonase (PG), an enzyme that degrades pectin in the plant cell wall, is highly active under acidic conditions. *S. sclerotiorum* produces five different types of PGs that are involved in infection establishment and lesion expansion (SSPG1) or tissue maceration (SSPG3, SSPG5 and SSPG6) (Li et al., 2004a, b). We have shown that *S. sclerotiorum* PGs alone can cause necrotic lesions to form (Dallal-Bashi et al., 2009a). A *S. sclerotiorum* line in which SSPG1 was disrupted has been generated. To protect themselves, plants produce

polygalacturonase inhibitor proteins (PGIPs) to inactivate these enzymes and we have isolated the genes for 17 *B. napus* PGIPs (Hegedus et al., 2008). As noted above, we are also determining which BnPGIPs inhibit the activity of *S. sclerotiorum* polygalacturonases. Five fungal polygalacturonases and four types of *B. napus* PGIPs were expressed in a yeast system and tagged for easy purification. The biochemical interaction between was examined and some found to have inhibitor activity. In transgenic *A. thaliana*, BnPGIP1 and BnPGIP2 provided resistance to the necrotizing effects of SSPG3 and SSPG6 (Figure. 9) and similar *B. napus* lines have now been made.

Under saprophytic growth conditions, *SsPG1d, SsPG 3, SsPG 5* and *Ssxpg1* expression was induced by pectin and galacturonic acid and subject to catabolite repression by glucose. Conditions could not be identified under which *SsPG 6* or *Ssxpg2* were expressed well. Transfer of mycelia from liquid media to solid substrates induced expression of *SsPG1d* suggesting that it may also be regulated by thigomotrophic interactions. Under pathogenic conditions, *SsPG1d* was highly expressed during infection. *SsPG3* was also expressed during infection, albeit at lower levels than *SsPG1d*, whereas *SsPG5, SsPG6* and *Ssxpg1* were expressed only weakly.

Contact with the waxy cuticle on the plant leaf surface is the first step in the infection process and induces endopolygalacturonase 1 (*SsPG1*) gene and cutinase (*SsCut1*) expression. We investigated the interplay between physical contact perception and carbon metabolism on *SsPG1* and *SsCut1* expression. Contact of mycelia with the plant leaf surface resulted in a strong induction of *SsPG1* and *SsCut* expression. Interestingly, glucose, which suppresses *SsPG1* induction in liquid culture, was found to enhance expression on the leaf surface. Thus, it seems that the inductive signal provided by either contact with the cuticle or components of the wax itself are able to over-ride the catabolite repressive effects of glucose. On leaves devoid of wax, *SsPG1* expression was induced only in the absence of glucose, supporting the notion that some feature of the wax cuticle is responsible for induction. In the absence of the cuticle, *SsPG1* induction is once again subject to repression by glucose. The response to contact with Parafilm, a solid hydrophobic surface, was the same as that of leaf stripped of cuticle. Therefore, some specific factor or property(s) associated with the leaf cuticle or its surface wax is required for

induction of *SsPG1* expression. Identifying these factors would allow for the development of *B. napus* lines lacking them and therefore able to prevent the very early stages of the infection.

III. *S. sclerotiorum* factors contributing necrosis

Successful host colonization by necrotrophic plant pathogens requires induction of plant cell death to provide nutrients needed for infection establishment and progression. The endo-PGs SSPG3 and SSPG6 caused the formation of necrotic lesions when infiltrated into leaves or when placed on leaves with an abraded cuticle. Leaves that had been stripped of cuticle were more effectively colonized by *S. sclerotiorum*.

We have also cloned two genes encoding necrosis and ethylene inducing peptides from *S. sclerotiorum* which we named *SsNep1* and *SsNep2*. Both peptides induce necrosis when expressed transiently in tobacco leaves (Figure 10). *SsNep1* is expressed at a very low level relative to *SsNep2* during the infection. The expression of *SsNep2* was induced by contact with solid surfaces and was expressed in both the necrotic zone and at the leading margin of the infection. *SsNep2* expression was dependent on calcium and cyclic AMP (cAMP) signaling as compounds affecting these pathways reduced or abolished *SsNep2* expression coincident with partial or total loss of virulence. An adenylate cyclase deletion mutant exhibited a similar phenotype.

V. Regulation of virulence gene expression

To better understand the signaling pathways involved in the response to surface contact we are also studying two key types of mitogen-activated protein (MAP) kinases, Bmp1 and Mkc1, that have been implicated in transduction of the surface sensing signal in *Candida albicans* and *Botrytis cinerea*. Such MAP kinases are expected to play an analogous role in the response of *S. sclerotiorum* to different surfaces. *Bmp1* MAP kinase expression was regulated by both carbon source and surface contact. Transfer of mycelia to normal or wax-stripped leaves had no effect on expression; however, levels were noticeably higher in the absence of glucose suggesting it is partially regulated by catabolite repression. Conversely, transfer to Parafilm strongly repressed expression of *Bmp1* and *Mkc1*. On the other hand, transfer to the surface of intact leaves induced *Mkc1* expression only in the presence of glucose, which was similar to that observed for *SsPG1*.

*S. sclerotiorum* lines in which *SsMkc1* was disrupted were found to be avirulent and unable to produce sclerotia indicating that this MAP kinase is important for both infection and development. MAP kinase activity is regulated by phosphorylation of specific serine and tyrosine residues. We have created a permanently active form of the Mkc1 MAP kinase that when introduced into *S. sclerotiorum* will allow us to identify the suite of virulence genes it regulates.

See the following appendices for additional details:

Dallal Bashi Z, Rimmer SR, Buchwaldt L, Khachatourians GG and Hegedus DD (2009a) Factors affecting the concerted expression of *Sclerotinia sclerotiorum cutinaseA* and *polyglacturonase1*. Mol. Plant Pathol. (in revision).

Dallal Bashi, Hegedus, D.D., Buchwaldt, L., Rimmer, S.R. and Borhan, M.H. (2010) Expression and regulation of *Sclerotinia sclerotiorum* necrosis and ethylene inducing peptides (NEPs). Mol. Plant Pathol. 11: 43-53.

Dallal Bashi Z, Khachatourians GG and Hegedus DD (2009c) Isolation of fungal homokaryotic lines from heterokaryotic transformants by sonic disruption of mycelia. Biotechniques 48: 351-354.

Li, R., Rimmer, R., Buchwaldt, L., Sharpe, A.G., Séguin-Swartz, G., and Hegedus, D.D. (2004a) Interaction of *Sclerotinia sclerotiorum* with *Brassica napus*: Cloning and characterization of endo- and exo-polygalacturonases expressed during saprophytic and parasitic modes. Fungal Genet. Biol. 41: 754-765.

Li, R., Rimmer, R., Buchwaldt, L., Sharpe, A.G., Séguin-Swartz, G., and Hegedus, D.D. (2004b) Interaction of *Sclerotinia sclerotiorum* with a resistant *Brassica napus* cultivar: Expressed sequence tag analysis identifies genes associated with fungal pathogenesis. Fungal Genet. Biol. 41: 735-753.

**Personnel:**

| Name | Position/Level | Salary | Source | Time |
|---|---|---|---|---|
| Diana Bekkaoui | Technician | $50,000 | ADF | 100 % |
| Zafer Dallal Bashi | Graduate Student | $25,000 | ADF | 100 % |
| Dr. Jianwei Zhao | PDF | $47,000 | SCDC/MII | 100 % |
| Shea Shirley | Summer Student | $13,000 | SCDC/MII | 100% |
| Sayna Bahrani | Casual Technician | $6,000 | SCDC/MII | 100% |

**Project Developed Materials:**

1. Database of genes expressed in *B. napus* cultivars resistant and susceptible to *S. sclerotiorum*.

Supplementary Table S1. List of differentially expressed genes in *B. napus* cv. Westar
Supplementary Table S2. List of differentially expressed genes in *B. napus* cv. ZY821
Supplementary Table S3. Comparison of inoculated *B. napus* cv. ZY821 and Westar

2. *B. napus* and/or *A. thaliana* lines expressing defense genes under the control of constitutive or inducible promoter.
    - *Hevein1*, *Hevein4* and *BnLectin*
    - *BnPGIP1* and *BnPGIP2*
    - *BnAnnexin2* and *BnAnnexin4*
    - *BnWRKY33*, *BnWRKY40* and *BnWRKY40*
    - Oxalate oxidase (alone and in combination with PGIP genes)
3. Genes associated with QTLs for resistance to sclerotinia
4. Lines of *S. sclerotiorum* with disrupted *SsPG1* or *SsMkc1* genes.

**Acknowledgements:** SCDC support of the project was acknowledged in the following:

**Publications:**

Dallal Bashi Z, Rimmer SR, Buchwaldt L, Khachatourians GG and Hegedus DD (2009) Factors affecting the concerted expression of *Sclerotinia sclerotiorum cutinaseA* and *polyglacturonase1*. Mol. Plant Pathol. (in revision).

Dallal Bashi Z, Khachatourians GG and Hegedus DD (2009c) Isolation of fungal homokaryotic lines from heterokaryotic transformants by sonic disruption of mycelia. Biotechniques 48: 351-354.

Dallal Bashi, Hegedus, D.D., Buchwaldt, L., Rimmer, S.R. and Borhan, M.H. (2010) Expression and regulation of *Sclerotinia sclerotiorum* necrosis and ethylene inducing peptides (NEPs). Mol. Plant Pathol. 11: 43-53.

Zhao J, Buchwaldt L, Rimmer SR, Sharpe A, Bekkaoui D and Hegedus DD (2009) Patterns of differential gene expression in *Brassica napus* cultivars infected with *Sclerotinia sclerotirum*. Mol. Plant Pathol. 10: 635-649.

Zhao J, Buchwaldt L, Rimmer SR, Brkic M, Bekkaoui D and Hegedus DD (2009) Differential expression of duplicated peroxidase genes in the allotetraploid *Brassica napus*. Plant Physiol. Biochem. 47: 653-656.

Hegedus DD, Li R, Buchwaldt L, Parkin I, Whitwill S, Coutu C, Bekkaoui D, Rimmer SR (2008) *Brassica napus* possesses an expanded set of polygalacturonase inhibitor protein genes that are differentially-regulated in response to *Sclerotinia sclerotiorum* infection, wounding and defense hormone treatment. Planta 228:241-253.

Hegedus DD and Rimmer SR (2005) *Sclerotinia sclerotiorum*: When “to be or not to be” a pathogen. FEMS Microbiology Lett. 251:177-184.

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Li R, Rimmer R, Buchwaldt L, Sharpe AG, Séguin-Swartz G and Hegedus DD (2004b) Interaction of *Sclerotinia sclerotiorum* with a resistant *Brassica napus* cultivar: Expressed sequence tag analysis identifies genes associated with fungal pathogenesis. Fungal Genet. Biol. 41: 735-753.

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**Expense Statement:** Attached

**Table 1.** Total number of genes that were differentially expressed in *B. napus* Zhongyou 821 and Westar stems after inoculation with *S. sclerotiorum*.

| Time (h) | Zhongyou 821 | | Westar | |
|---|---|---|---|---|
| Post- Infection | Up | Down | Up | Down |
| 6 | 18 | 0 | 0 | 0 |
| 12 | 286 | 2 | 54 | 0 |
| 24 | 624 | 245 | 1311 | 1655 |
| 48 | 2161 | 2614 | 2237 | 2597 |
| 72 | 2091 | 2304 | 1458 | 2002 |

**Table 2.** Genes up-regulated in *B.napus* Zhong You 821 stems within 6 hours after inoculation with *S. sclerotiorum*.

| Oligo ID | Fold Change | P-value | *A. thaliana* locus | Description |
|---|---|---|---|---|
| BN24266 | 7.2 | 7.69E-09 | At2g43590 | chitinase |
| BN27647 | 5.2 | 2.32E-09 | At3g16720 | zinc finger (C3HC4-type) protein |
| BN25790 | 5.1 | 9.73E-10 | At3g16530 | legume lectin |
| BN25791 | 4.1 | 5.53E-07 | At3g16530 | legume lectin |
| BN19870 | 3.7 | 6.38E-07 | At1g21100 | O-methyltransferase |
| BN17285 | 3.7 | 9.21E-07 | At2g38470 | WRKY family transcription factor |
| BN16077 | 3.4 | 1.29E-06 | At4g09030 | arabinogalactan-protein (AGP10) |
| BN16554 | 3.3 | 1.34E-06 | At1g70170 | matrixin |
| BN24387 | 3.2 | 3.33E-06 | At1g61360 | S-locus lectin protein kinase |
| BN17170 | 2.8 | 3.21E-07 | At1g14870 | expressed protein |
| BN27200 | 2.8 | 2.35E-06 | At4g11650 | osmotin-like protein (OSM34) |
| BN14604 | 2.7 | 3.27E-09 | At2g24810 | thaumatin |
| BN15694 | 2.4 | 8.85E-06 | At2g30020 | protein phosphatase 2C |
| BN20480 | 2.4 | 1.49E-07 | At5g63140 | calcineurin-like phosphoesterase |
| BN14832 | 2.3 | 1.05E-06 | At2g22500 | mitochondrial substrate carrier |
| BN22453 | 2.2 | 4.38E-06 | At1g74100 | sulfotransferase |
| BN18515 | 2.1 | 2.05E-06 | At1g32350 | alternative oxidase |
| BN14925 | 2.1 | 1.61E-06 | At3g57240 | beta-1,3-glucanase (BG3) |

**Table 3.** Genes induced only in *B. napus* ZY821 stems after *S. sclerotiorum* infection.

| Oligo IDa | Corresponding *A. thaliana* locus | Description | Fold Increase 6 hour | 12 | 24 | 48 | 72 |
|---|---|---|---|---|---|---|---|
| **Transcription** | | | | | | | |
| BN11472 | At3g57390 | MADS-box protein | - | 4.16c | 6.28 | 12.8 | 4.95 |
| BN26939 | At2g47460 | Myb transcription factor | - | 2.21 | 2.17 | 5.28 | 5.71 |
| BN27665 | At1g28360 | ERF domain protein 12 | - | - | 3.05 | 9.46 | 7.87 |
| BN26396 | At5g57520 | Zinc finger | - | - | 3.30 | 5.37 | 3.57 |
| **Stress** | | | | | | | |
| BN25790 | At3g16530 | Lectin | 5.18 | 15.8 | 50.5 | 109 | 108 |
| BN22148 | At3g57260 | Endo beta-1,3-glucanase | 2.27 | 3.52 | 6.11 | 9.91 | 7.56 |
| BN18917 | At2g38750 | Annexin | - | 3.65 | 2.66 | 25.1 | 33.5 |
| BN27037 | At4g22990 | Multidrug resistance protein 2 | - | 2.20 | 2.57 | 8.02 | 9.01 |
| BN22038 | At4g34135 | UDP-glucosyl transferase | - | - | 3.95 | 8.95 | 3.40 |
| BN16777 | At4g15160 | Protease inhibitor | - | - | 2.92 | 3.02 | 18.4 |
| BN20902 | At4g27670 | Heat shock protein | - | - | 2.65 | 7.35 | 3.71 |
| BN11639 | At1g69530 | Expansin | - | - | - | 7.21 | 9.46 |
| BN17923 | At4g10265 | Wound-responsive protein | - | - | - | 6.03 | 7.79 |
| BN18404 | At5g27060 | Leucine rich-repeat | - | - | - | 5.58 | 5.00 |
| BN15878 | At5g65020 | Annexin | - | - | - | 4.26 | 7.39 |
| BN13492 | At2g37640 | Expansin | - | - | - | 3.90 | 7.79 |
| BN12243 | At5g06860 | PGIP1 | - | - | - | 3.27 | 18.9 |
| **Metabolism** | | | | | | | |
| BN22522 | At4g27070 | Tryptophan synthase | 2.65 | 2.69 | 4.65 | 10.6 | 8.31 |
| BN24404 | At4g15417 | Ribonuclease lll | 2.00 | 2.76 | 3.33 | 26.5 | 14.0 |
| BN23990 | At1g05680 | UDP-glucosyl transferase | - | 2.53 | 2.95 | 24.5 | 9.04 |
| BN16989 | At1g48920 | Nucleolin | - | 2.08 | 4.04 | 6.67 | 4.41 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| BN18865 | At1g07720 | Beta-ketoacyl-CoA synthase | - | - | 3.09 | 28.8 | 15.7 |
| BN15489 | At1g17710 | Phosphoric hydrolase | - | - | 3.01 | 73.0 | 54.4 |
| BN23164 | At4g18950 | Ankyrin protein kinase | - | - | 2.10 | 5.80 | 4.61 |
| **Others** | | | | | | | |
| BN14638 | At1g19770 | Purine permease | 2.13 | 2.10 | 4.59 | 10.9 | 10.1 |
| BN20514 | At1g76790 | *O*-methyltransferase | 2.00 | 7.87 | 12.5 | 25.3 | 31.1 |
| BN13257 | At1g54020 | Lipolytic enzyme | - | 3.15 | 14.2 | - | - |
| BN14979 | At2g30540 | Glutaredoxin | - | 2.16 | 4.74 | 17.4 | 7.81 |
| BN13468 | At4g22620 | Auxin-induced protein | - | 2.05 | 2.16 | - | 8.11 |
| BN26740 | At2g31990 | Exostosin | - | 2.04 | 2.17 | 10.4 | 7.10 |
| BN23610 | At5g43340 | Phosphate transporter | - | - | 4.66 | 27.5 | 14.8 |
| BN20039 | At1g15180 | Membrane protein family | - | - | 4.37 | 11.7 | 12.1 |
| BN18437 | At5g15440 | Circadian clock factor | - | - | 3.75 | 13.4 | 11.5 |
| BN24154 | At5g01210 | Transferase | - | - | 3.63 | 5.48 | 7.55 |
| BN17385 | At5g10300 | Hydrolase | - | - | 2.14 | 3.91 | 7.93 |
| BN26419 | At5g17450 | Copper homeostasis factor | - | - | - | 11.1 | 9.69 |
| BN19106 | At5g01750 | Expressed protein | - | - | - | 9.41 | 6.90 |
| BN20931 | At5g60760 | 2-phosphoglycerate kinase | - | - | - | 7.38 | 4.21 |
| BN24358 | At3g54040 | Photoassimilate protein | - | - | - | 5.07 | - |
| BN17022 | At3g13857 | Expressed protein | - | - | - | 2.94 | 10.5 |
| BN13468 | At4g22620 | Auxin-induced protein | - | - | - | - | 9.38 |

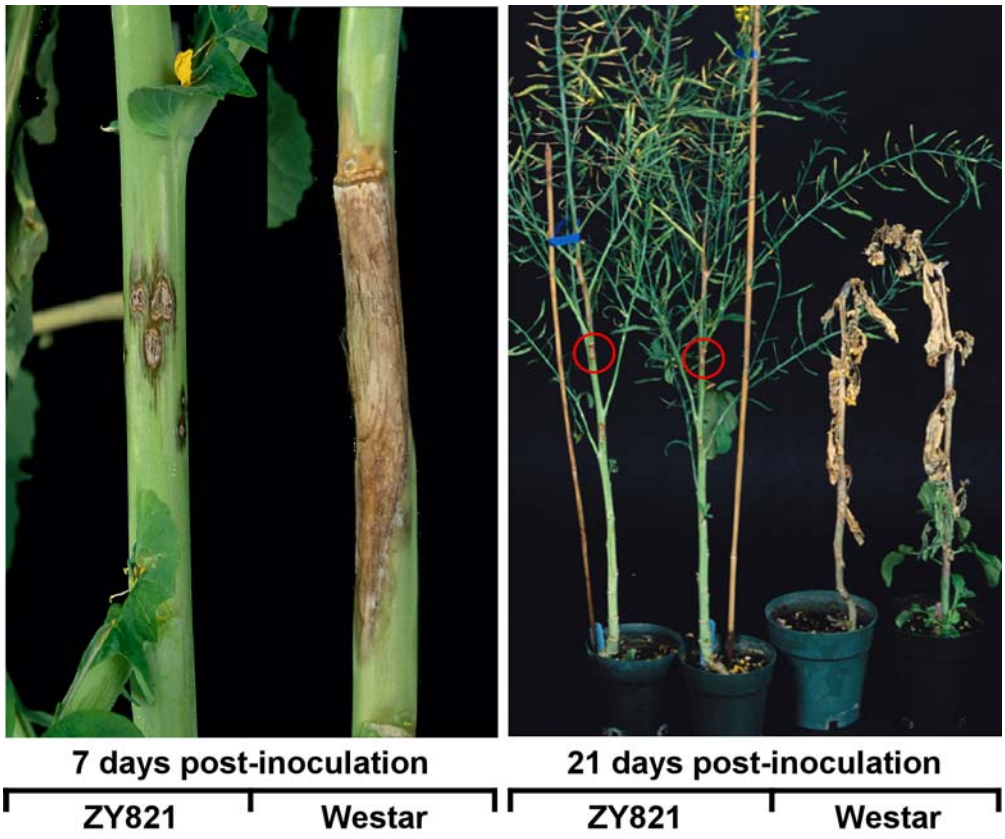


**Figure 1.** Phenotypes of *B. napus* ZY821 and Westar 21 days after inoculation of stems with *S. sclerotiorum*.

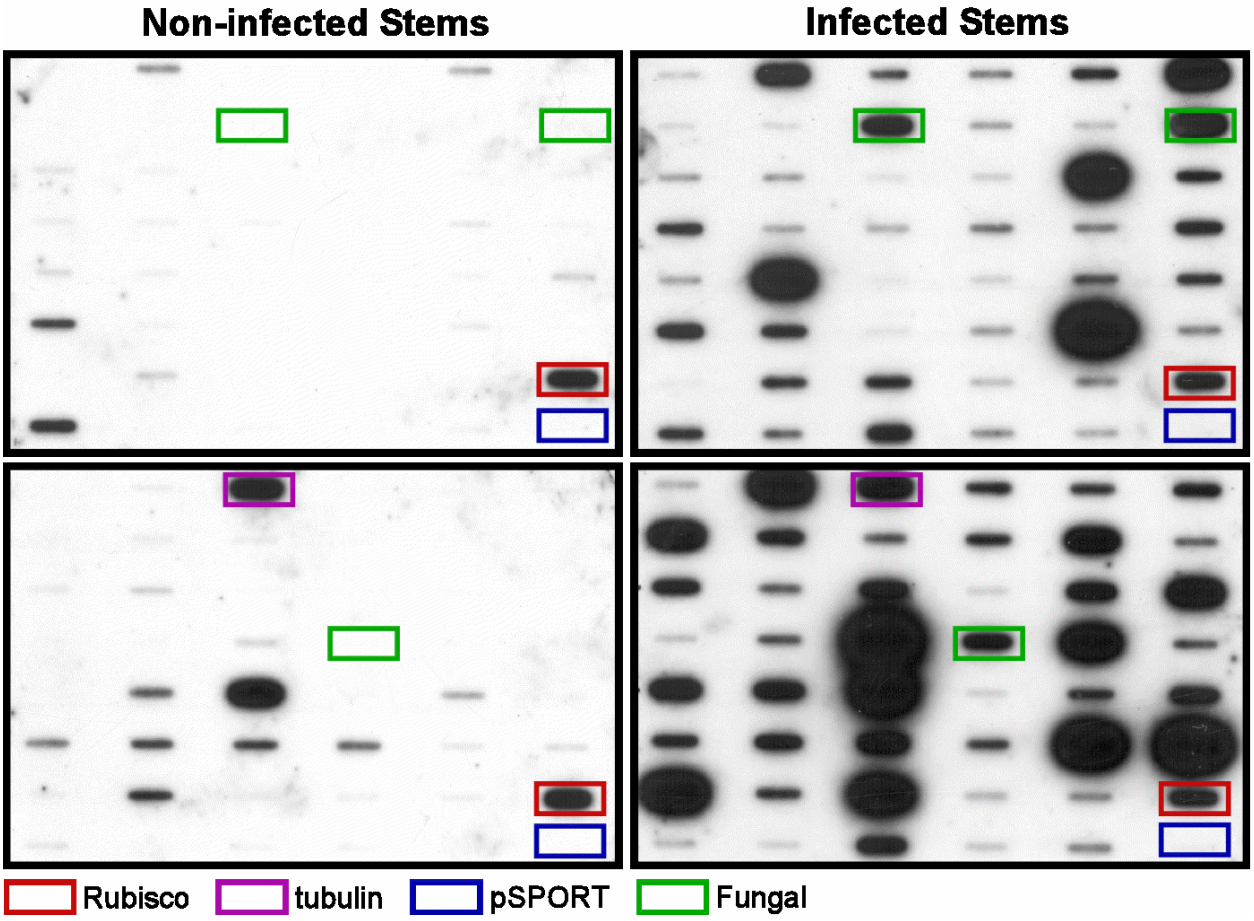


**Figure 2.** Reverse Northern blot analysis of a cDNA macro-array showing numerous genes expressed in *B. napus* ZY821 stems after inoculation with *S. sclerotiorum* compared to non-infected stems. Rubisco and tubulin genes are used for comparison. pSport serves as a negative control and fungal genes as positive controls for infection.

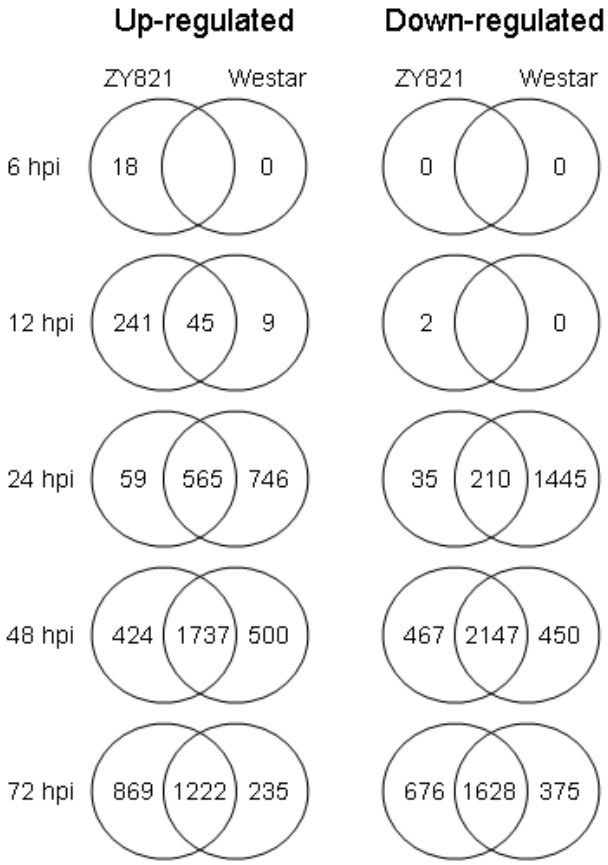


**Figure 3.** Venn diagrams showing the number of differentially-regulated genes that were common and unique to *B. napus* ZY821 or Westar 6 – 72 h after inoculation of stems with *S. sclerotiorum*. Genes were identified by comparing each time point using one sample t-test with a false discovery rate (FDR) of 0.005 and cut-off value of 2-fold change.

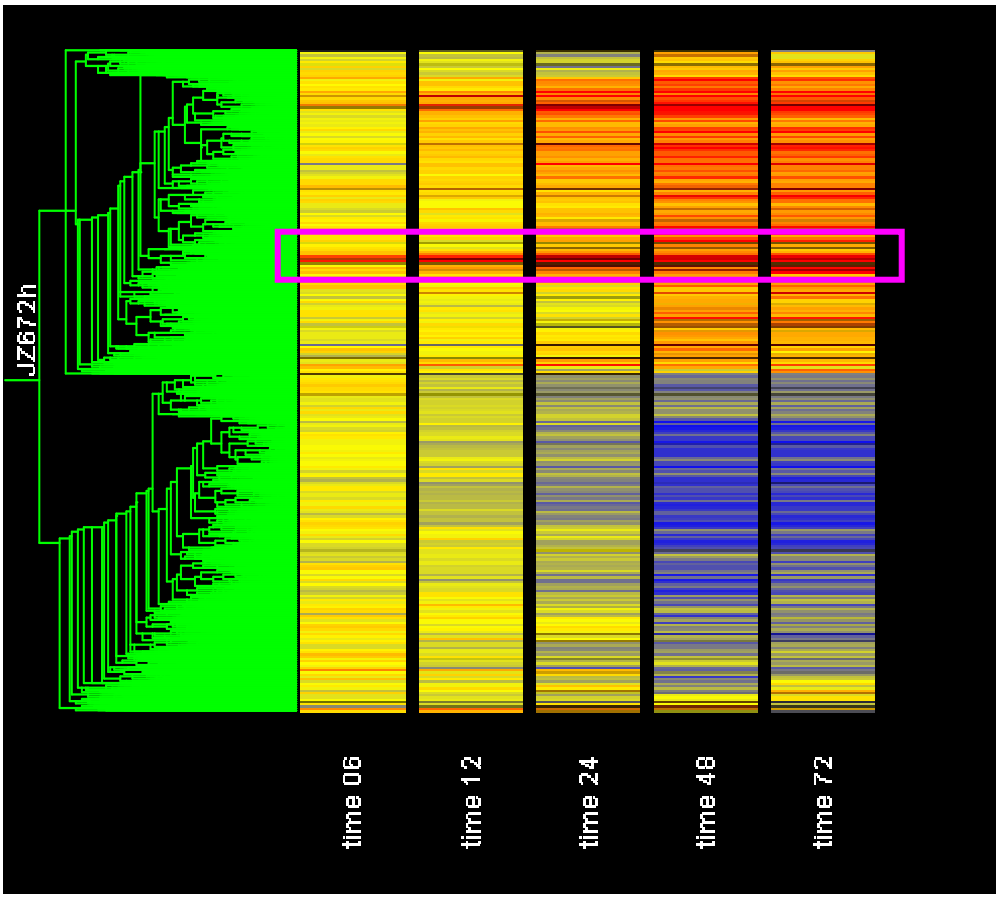


**Figure 4.** Dendogram showing clusters of *B. napus* ZY821 genes that were up- (red) or down- (blue) regulated at various times post-inoculation with *S. sclerotiorum*. A yellow bar indicates that no alteration in expression was observed at the specific time point. The boxed area (purple) indicates the subset of genes induced at the very early stages of the infection that are being pursued as potential candidates for developing resistance.

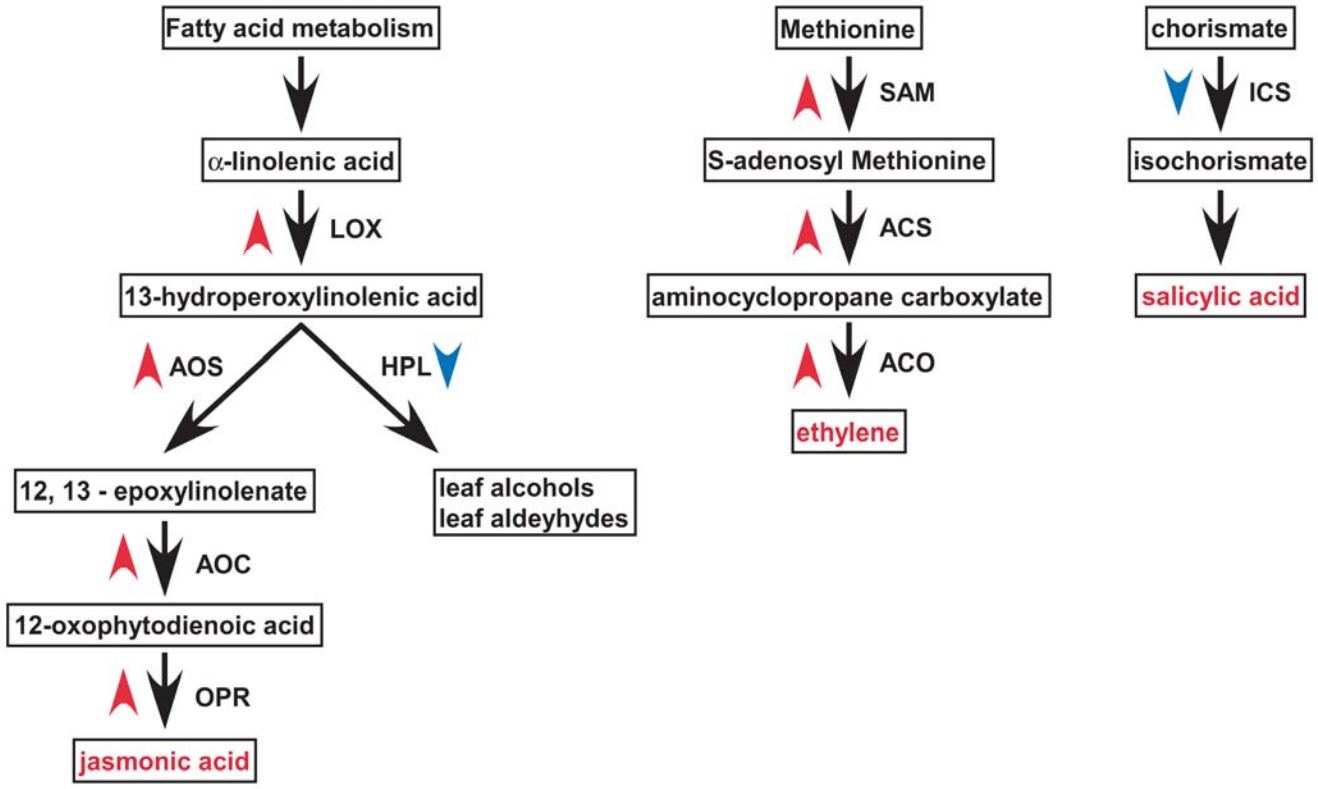


**Figure 5.** Increase (red arrows) and decrease (blue arrows) in the expression of genes in hormone biosynthetic pathways observed in *B. napus* tissue infected by *S. sclerotiorum.*

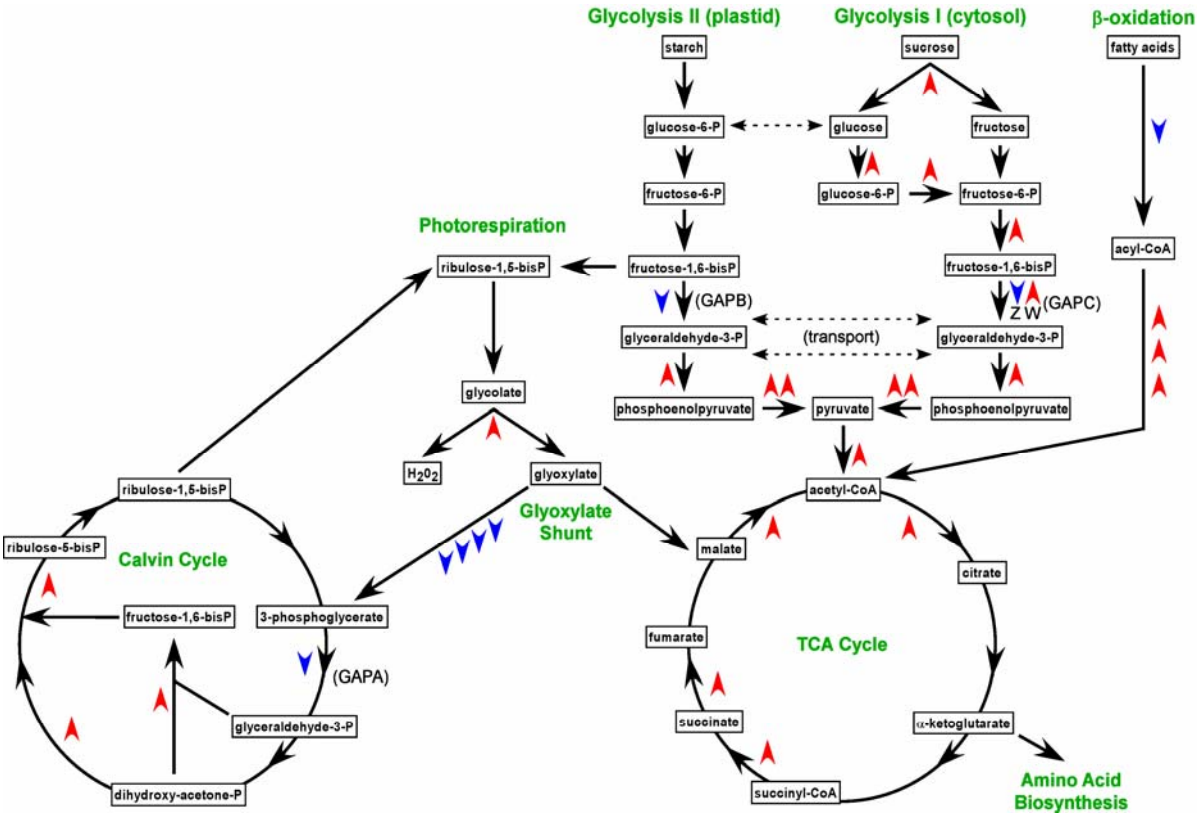


**Figure 6.** Expression of genes involved in carbon metabolism in *B. napus* ZY821 (Z) and Westar (W) after *S. sclerotiorum* inoculation. Increase (red arrows) and decrease (blue). Glyceraldehyde-3-phosphate dehydrogenase isoforms (GAP).

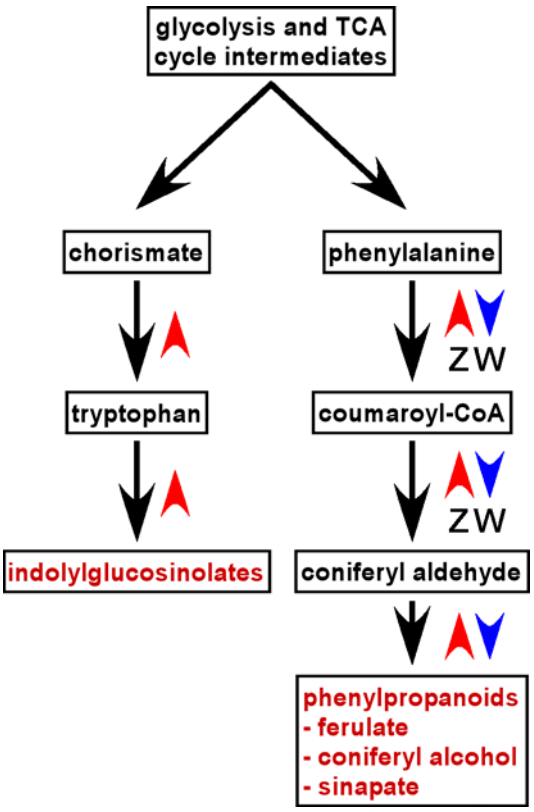


Figure 7. Expression of genes involved amino acid-derived secondary metabolites in *B. napus* ZY821 (Z) and Westar (W) after *S. sclerotiorum* inoculation. Increase (red arrows) and decrease (blue).

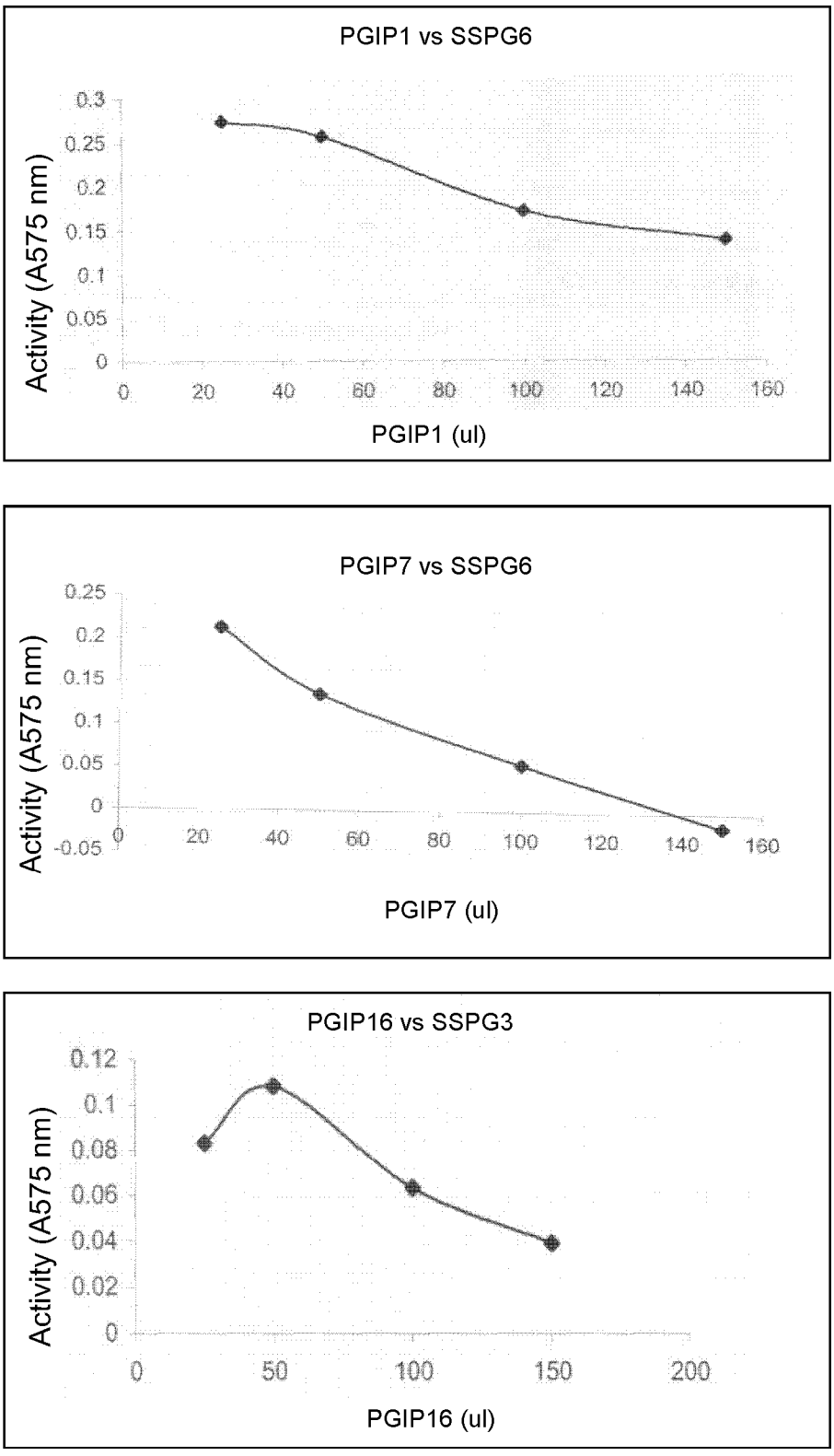


**Figure 8.** Inhibition of *S. sclerotiorum* SSPG activity by *B. napus* PGIP.

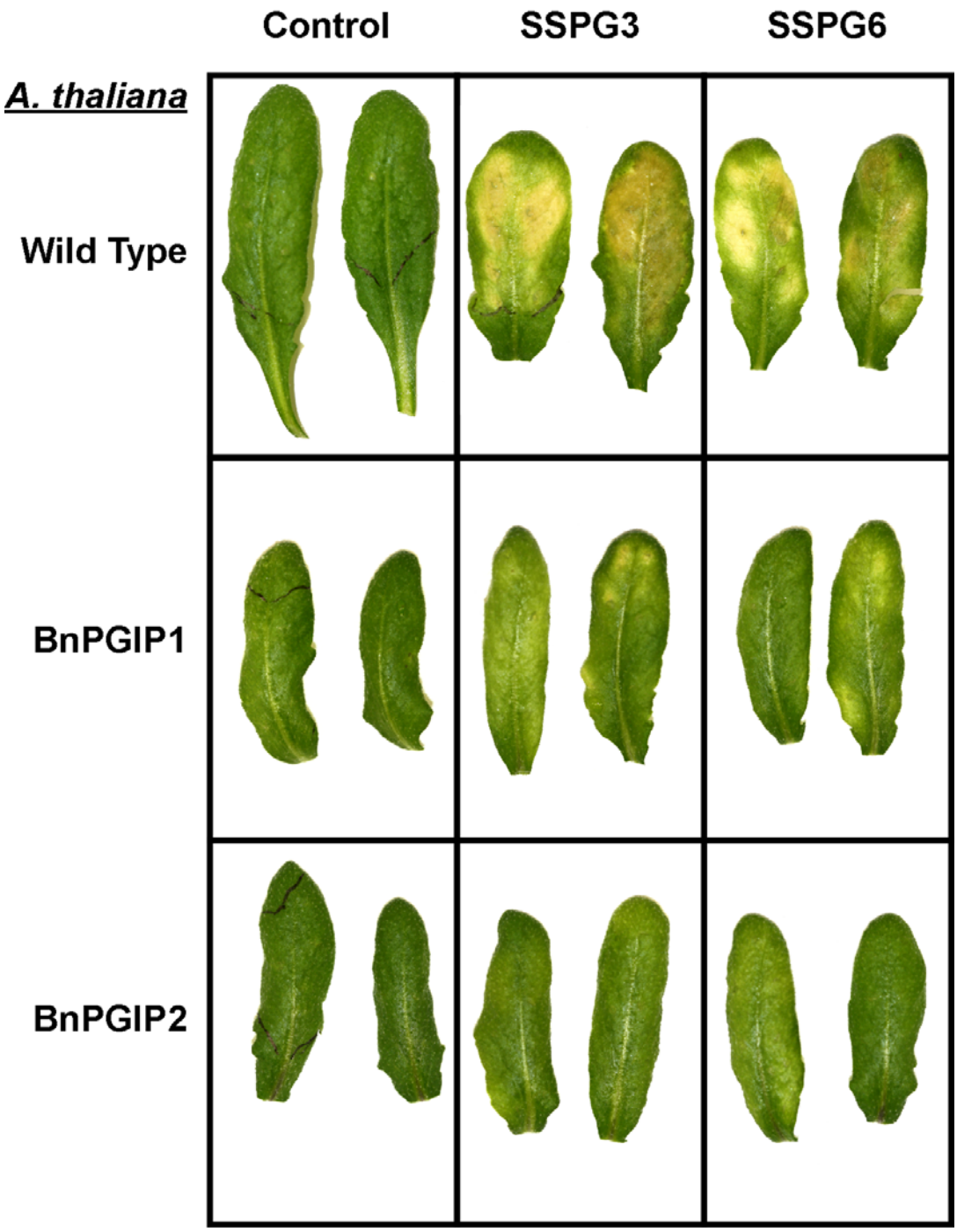


**Figure 9.** Symptoms arising after 6 days after infiltration of the leaves of an *A. thaliana* wild type lines or lines expressing *B. napus* polygalacturonase inhibitor proteins (BnPGIP1 and BnPGIP2) with *S. sclerotiorum* polygalacturonases (SSPG3 and SSPG6). Control leaves were infiltrated with buffer.

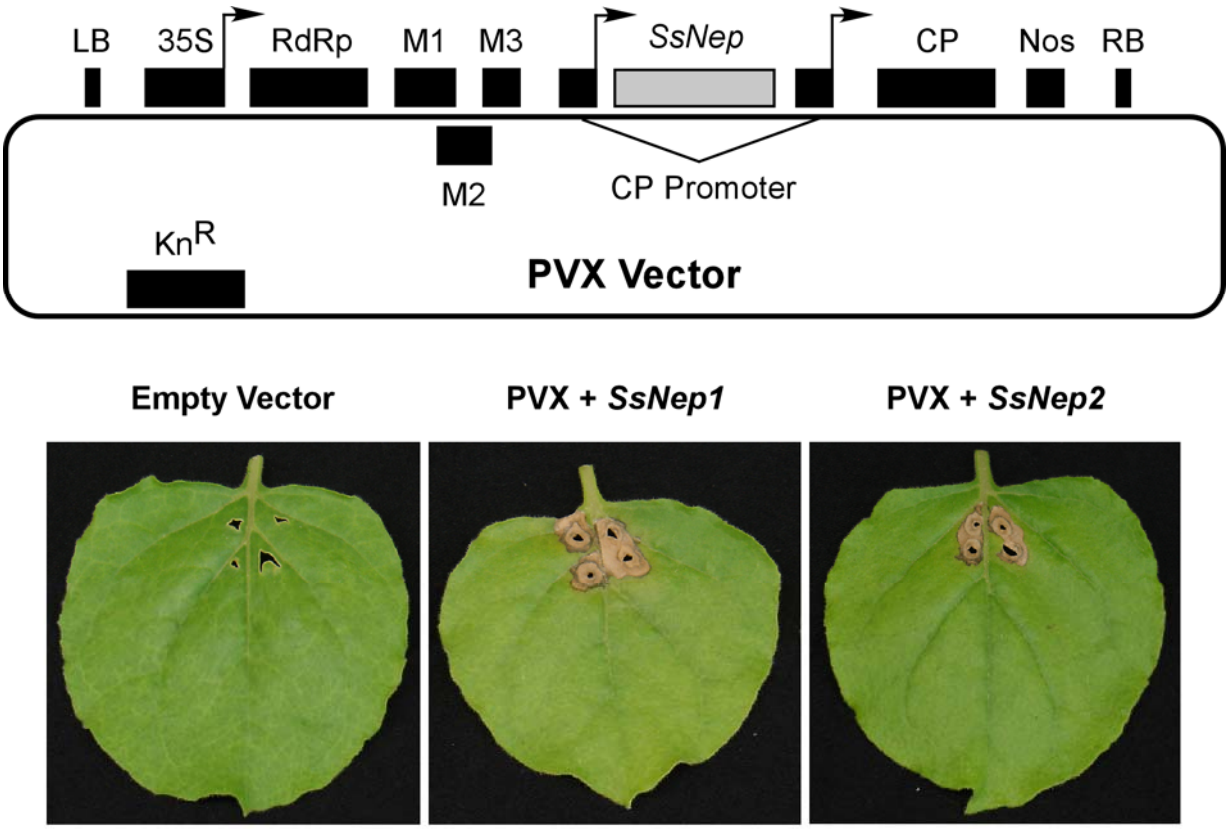


**Figure 10.** Map of the potato virus X-based binary vector pgR107 used for the in planta expression of SsNep genes. Elements shown are: LB, left border; 35S, cauliflower mosaic virus 35S promoter; RdRp, viral RNA-dependent RNA polymerase; M1-M3, viral movement proteins; CP, coat protein, Nos, *Agrobacterium tumefaciens* nopaline synthetase transcriptional terminator; RB, right border. Necrotic symptoms observed on *Nicotiana benthamiana* leaves two weeks after inoculation of leaves with PVX virus, PVX + *SsNep1* or PVX + *SsNep2*.