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Assessments of novel transgenic germplasm and treatment with the phytohormone cytokinin for reducing feeding damage by flea beetles, *Phyllotreta* spp. (Coleoptera: Chrysomelidae) in canola

by

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Abstract

Flea beetles [*Phyllotreta cruciferae* (Goeze) and *Phyllotreta striolata* (Fabricius)] (Coleoptera: Chrysomelidae) pose significant threats to early canola seedling establishment. The resistance potential of transgenic *Brassica napus* expressing cDNA encoding pea *PR10.1* and *10.4 (ABR17)* in Westar and DH19 backgrounds, respectively, was investigated. In addition, the effects of treatments with different concentrations of the cytokinin 6-benzylaminopurine (BAP) and with Helix® (thiamethoxam insecticide) on flea beetle tolerance were investigated. Our results indicate that transgenic lines expressing *PR10.1* and *PR10.4* suffered the least damage from *P. cruciferae* when compared with Helix®-treated doubled haploid or Westar genotypes, while cytokinin treatments conferred some tolerance. *P. striolata* experiments demonstrated that insecticide treatments were most effective for reducing damage. Helix® treatments displayed the most significant beetle mortality while neither *PR10.1*, *PR10.4* nor cytokinin treatments demonstrated insecticidal activity. Proteomic analysis revealed significant increases in abundance of some proteins including protease inhibitors in *ABR17* seedlings damaged by herbivory.

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Chapter 1. Introduction

1.1 Canola and Canada

Modified oilseed rape plants, commonly known as canola, were developed by Canadian plant scientists in the 1970s. Canola has become a valuable cash crop for the Canadian economy, generating over $15 billion annually in economic revenue (Canola Council of Canada 2011). In 2006, 9.1 million tonnes of seed were produced in Canada, at an average yield of 2050 kg/ha and average oil content of 42.5% (Canola Council of Canada 2011). In 2008-2009, China represented Canada's greatest importer of canola seed with 2.87 million tonnes accounting for $1.3 billion (Canola Council of Canada 2011). The oil is extracted from the plant's seed and the remainder is used as meal for animal feed, thus increasing canola's versatility and economic value. Canola is comprised of *Brassica napus* L. and *Brassica rapa* L., which belong to the family Brassicaceae alongside cabbage (*Brassica oleracea* L. var capitata), brown mustard, (*Brassica juncea* (L.) Czern), white mustard (*Sinapis alba* L.), and several other economically important crop species (Downey 1983).

Oilseed rape or canola is capable of growing at relatively low temperatures, and requires fewer heat units than other oilseed crops, making it a good candidate for growth in extreme environments, such as the Canadian prairies (Thomas 2002). What made rapeseed oil so attractive was its ability to stay on water and steam-washed metal surfaces better than other lubricants (Anonymous 1982). The western prairies are the focal point for canola growth, and depending on the year, canola constitutes at least the third largest crop in terms of area under production. In the years 2007-2010, canola farm production and oilseed crushing have had the largest economic impact agriculturally in the prairie provinces valued at approximately $13.7

billion annually, with Saskatchewan, Alberta and Manitoba contributing $5.4, $5.0 and $3.3 billion respectively (Canola Council of Canada 2011).

One of the barriers that initially had to be overcome with transforming rapeseed to an edible crop for humans and animals revolved around its glucosinolate and erucic acid contents. Although rapeseed meal was high in protein and amino acids, the pungent odor and biting taste attributed to glucosinolates limited its palatability for use as animal feed. Erucic acid is a monosaturated omega -9- fatty acid, which is toxic to humans in large doses. Glucosinolates can cause nutritional problems and reduced uptake during feed formulation and utilization, since glucosinolates are converted to other compounds (Downey 1983). Plant breeders and geneticists have successfully reduced the concentrations of both glucosinolate and erucic acid in the seed, creating "double low" canola cultivars (Anonymous 1982).

1.1.1 Secondary Compounds and Insect Interactions

Chemical compounds given off by plants allow insects to locate their hosts. These signals may promote herbivory or influence predatory activity depending on the situation. Some research has been conducted on the host-locating ability of the crucifer flea beetle, *Phyllotreta cruciferae* (Goeze) (Coleoptera: Chrysomelidae), yet much is still unknown (Gruber et al. 2009). Brassicaceae produce crucifer-specific glucosinolates (GSs), volatile isothiocyanate (ITC) and nitrile degradation products (Pivnick et al. 1992). Several factors, known and unknown, affect a plant's ability to use GSs as an herbivory attractant or deterrent. Myrosinase-binding, epithiospecifier proteins and many other unknown proteins determine the role of GS as an insect stimulus (Lambrix et al. 2001). For instance, the ρ-hydroxybenzyl-GS metabolite conveys resistance to *P. cruciferae* in *Sinapis alba* L. (Soroka et al. 2003), yet demonstrates no added resistance when produced in *Arabidopsis thaliana* L. (Nielson et al. 2001). The first instance of a

volatile compound being used to affect insect activity and control was when a terpene was used to draw the peach potato aphid *Myzus persicae* (Sulz.) (Heteroptera: Aphididae) to come into contact with a myco-insecticide (Roditakis et al. 2000).

1.2 *Phyllotreta* spp. and Their Impact on Canola

The flea beetles, *P. cruciferae* and *Phyllotreta striolata* (Fabricius) (Coleoptera: Chrysomelidae), are important phytophagous specialist pests of the Brassicaceae family. Their host plants include flixweed, wild mustard, cabbage and the most economically important host, canola. The adults overwinter in stubble or leaf litter and emerge in the spring to feed on volunteer canola and seedling crucifers (Burgess 1977; Ulmer and Dosdall 2006; Gruber et al. 2009). Five species of flea beetle have been identified on the Canadian prairies, with *P. cruciferae* being the most significant and prominent (Westdal and Romanow 1972; Burgess 1977). *Phyllotreta striolata*, *Phyllotreta albionica* (LeConte), *Phyllotreta robusta* LeConte, and *Psylliodes punctata* Melsh comprise the other known species. *Disonycha triangularis* (Say), and species of *Crepidodera* and *Chaetocnema*, are also found throughout the prairies but do not represent pest species (Burgess 1977). *Phyllotreta cruciferae* and *P. striolata* represent the dominant pest species; however, *P. punctata* is being found on a more regular basis (L. Dosdall, personal communication). Little research has been conducted on the biology of *P. punctata*, so its impact as a significant pest species has not yet been established. Feeding by adult beetles can result in seedling mortality, delayed maturity, and consequently lower yields (Lamb 1988b). Flea beetles are responsible for causing economic losses of more than $300 million dollars annually in western Canada (Madder and Stemeroff 1988).

Introduced from Europe to the North American west coast in the early 1920's, the first flea beetle specimen of *P. cruciferae* (initially recorded as *Phyllotreta columbiana* Chittenden but later synonomized under *P. cruciferae*), was collected in British Columbia in 1923 (Milliron 1953). Damage to prairie brassicaceous crops during the 1930s and 1940s was likely due to *P. cruciferae* as a specimen was collected from Winnipeg, Manitoba in 1936. There may have been a second introduction during the 1940's as specimens were collected from the eastern United States, as it appeared to migrate to Ontario then the Maritimes (Westdal and Romanow 1972).

The holometabolous *Phyllotreta* life cycle entails an overwintering adult stage which takes shelter in leaf litter, soil stubble and shelterbelts. Adult beetles are striped or black with a metallic or bright blue luster at roughly 2 to 3 mm in length (Government of Manitoba 2010). Emergence occurs in the spring throughout the month of May as periods of 15°C weather persist (Burgess 1977; Ulmer and Dosdall 2006). When temperatures are too low for flight, emerging beetles walk into fields and adults feed on the leaves and stems of volunteer canola and brassicaceous weeds before moving onto canola seedlings and other cruciferous crops. Females deposit their small (~0.4 mm), elongated, yellow eggs at the base of host plants in early June as the adults begin dying off. In July the dirty-white colored larvae with brown heads hatch and feed on the root hairs of host plants for three to four weeks, causing relatively minor damage to plant growth and development (Government of Manitoba 2010). Mature larvae are up to 6 mm in length with three pairs of short legs on the thorax while the pupae are white with a length of ~2.4 mm (Government of Manitoba 2010). The larvae pupate in earthen cells and emerge as adults in late July and early August, and begin feeding on host plants to build up their fat reserves for overwintering. They continue to feed on the leaves and stems while also feeding on the siliques of host plants throughout August, until September when they begin migrating to overwintering sites (Feeny et al. 1970). Flea beetles are particularly active under warm, dry and

wind-less conditions. Typically only two generations of adult flea beetles appear in a single field season, including the overwintered adults in May followed by adults of the new generation in August to September (Burgess 1977; Ulmer and Dosdall 2006).

Phyllotreta cruciferae and *P. striolata* comprise the primary flea beetle species that have been studied in western Canada and were investigated in experiments described in this thesis. They are morphologically quite distinct and easy to distinguish from the color of their integument. *Phyllotreta cruciferae* has a sheen, metallic black or green integument. *Phyllotreta striolata*, introduced from Eurasia prior to 1801, has two very distinct yellow-orange stripes running longitudinally down the elytra (Burgess 1977). Males have short, pointed abdominal points while the females have longer, rounded abdomens. *Phyllotreta striolata* are much more voracious in terms of crop damage per individual, yet this species is generally much less abundant (Tansey et al. 2008). *Phyllotreta cruciferae* are found in great abundance in and around surrounding Edmonton farms. *Phyllotreta striolata*, although present, are much harder to find and less abundant (Sultani, personal observations).

1.2.1 Flea Beetle Control

Flea beetles chew small holes in leaves rendering a “shot-hole” appearance (Government of Manitoba 2010). Scouting techniques can include counting the number of infested plants per square meter and counting the number of feeding holes on collected leaves. The simplest technique is to rank a percentage of representative samples of plants based on cotyledon damage, the economic threshold being 25% of leaf tissue destroyed or damaged by adult feeding (Government of Manitoba 2010). Flea beetle damage can cause thinning the plant stand, delayed plant development and reduced seed quality and concentration. Although canola stands can withstand significant leaf area destruction without significant yield loss, a severe

infestation is capable of killing seedlings and destroying the entire crop. At the three- to four-leaf stage, plants are generally established enough to outgrow flea beetle damage (Government of Manitoba 2010).

Flea beetle densities observed during the fall season can help to determine densities in the next spring, and the risk of crop damage. The majority of canola growers rely on insecticide-coated canola seeds to protect seedlings from flea beetle attack. The coated seeds germinate in spring, and insecticide is taken up systemically to seedling tissues. Adults that feed upon the cotyledons and stems of the seedlings then die in the early season. Studies investigating the efficacy of the systemic neonicotinoid seed treatments, such as thiamethoxam and imidacloprid, have determined that *P. striolata* is more resistant than *P. cruciferae* (Tansey et al. 2008, 2009). *Phyllotreta cruciferae* experienced greater mortality and caused less feeding damage than *P. striolata* to the treated seedlings. Among the seed treatments assessed, fipronil, a contact, ingested and systemic GABA antagonist (Nauen and Bretschneider 2002) demonstrated greater efficacy than the others (Tansey et al. 2009). In spite of the advantages gained from insecticide-treated seeds over foliar sprays, it must be kept in mind that seed treatments are responsible for enormous additions of insecticides in western Canadian agroecosystems. Such insecticide additions to agricultural soils can be harmful to beneficial species such as soil microarthropods that enhance decomposition and nutrient cycling.

Although flea beetle predation has been observed for larvae of the lacewing, *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), and parasitism of adult beetles has been recorded for the braconid wasp, *Microctonus vittatae* Muesebeck (Hymenoptera: Braconidae), the overall efficacy of these natural enemies has been inadequately studied (Dosdall and Mason 2010). Furthermore, flea beetles emerge in large numbers over a short time thus overwhelming predators (Government of Manitoba 2010). The Holarctic, univoltine *M.*

vittatae is capable of parasitizing both *P. cruciferae* and *P. striolata* with a preference for *P. striolata* (Loan 1967; Wylie 1984). Studies determined that *P. striolata* adults were parasitized at a rate of 46%, while *P. cruciferae* were parasitized at 10% (Smith and Peterson 1950; Loan 1967). Females of *M. vittatae* oviposit in the flea beetle thorax, and subsequently the eggs absorb the host's body fat increasing in size by hundreds of times (Smith and Peterson 1950). Despite several eggs being laid, only one larva survives, feeding on host adipose tissue and amniotic cells of the egg, causing host sterility. The other *M. vitattae* larvae die off from secretions and cannibalism of its brethren (Smith and Peterson 1950; Loan 1967). The larvae undergo five instars prior to leaving the host, causing death.

Seeding into near-freezing soil in fall rather than more typical soils during spring can enable seeds to remain dormant during winter and germinate early in spring. Early seedling emergence and growth enables seedlings to progress beyond the vulnerable cotyledon stage before flea beetles invade (Dosdall and Stevenson 2005). Fall-seeded canola may grow to the three- to four-leaf stage by the time flea beetles emerge from overwintering, compared to the significantly more susceptible cotyledon stage of spring-seeded canola. Dosdall and Stevenson (2005) observed that fall-seeded canola may have damage ratings as high as 30% of the leaf tissue being consumed, while spring-seeded canola may have damage as high as 70%. By the time of flea beetle emergence, fall-seeded canola has shed dependence on its cotyledons for growth and development, unlike spring-seeded canola which remains dependent on them until later in the season (Dosdall and Stevenson 2005). An issue that arises with fall-seeding is that chemical polymer compounds, such as Extender® which can be coated onto the seed, are used to prevent the seeds from imbibing water and germinating. Weather patterns are never consistent between the years and an unseasonable warm spell during the winter months may result in the Extender® polymer breaking down and seeds germinating. A return of the frost

would then destroy the germinating seedling population and result in a greatly reduced plant stand during the spring season. Similarly, planting in early spring versus later spring may have a similar effect depending on the emergence timing of the flea beetle population. Should the flea beetles emerge earlier in spring, the early spring planting would have little effect. However, should the early spring-seeded canola seedlings develop for a more extended period before flea beetle emergence, they may be able to better withstand flea beetle attack. Although risky, fall-seeded canola demonstrates reduced susceptibility of seedlings to flea beetle attack relative to spring-seeding of canola (Dosdall and Stevenson 2005).

Widening row spacing and seeding at a greater density have also been shown to reduce flea beetle damage (Dosdall et al. 1999; Dosdall and Stevenson 2005). By increasing the seeding rate from 7.5 to 10.0 kg/ha or 12.5kg/ha, damage per plant was shown to be significantly reduced. In addition to the benefit that arises from increased seeding rate for the cultural control of flea beetles, this strategy also has the ability to manage root maggot damage and losses due to weeds (Dosdall et al. 1996; Dosdall and Stevenson 2005). Increasing the row spacing from 10 cm to 20 or even 30 cm also resulted in decreased plant damage (Dosdall et al. 1999). This may be caused by a flying insect's dependence on the visual contrast between vegetation and soil background (Ishii-Eiteman and Power 1997). Wider row spacing increases the visual contrast resulting in reduced susceptibility to flea beetle attack (Dosdall et al. 1999). Similarly, seeding *B*. *napus* and *B*. *rapa* with larger seeds produces seedlings of greater biomass that are more tolerant of flea beetle feeding (Elliott et al. 2007, 2008). By seeding larger seeds at a higher density, the need for insecticidal usage is dramatically reduced along with its environmental impact.

The implementation of intercropping has been able to maintain low populations of flea beetles compared to vast monocultures. This is likely due to chemical and physical interference

with respect to host-plant locating and fewer acceptable environments (Pimentel 1961; Tahvanainen and Root 1972). In Alberta, intercrops of canola and wheat were sometimes found to result in decreased feeding damage to canola by flea beetles (Hummel et al. 2009). A reduced or zero tillage regime has also been recommended for reducing insecticide use against flea beetles as it provides canola seedlings with cooler and moister conditions relative to conventional tillage (Dosdall et al. 1999). *Phyllotreta* spp. prefer warm, dry conditions (Tahvanainen 1972), so populations can show a drastic reduction with reduced tillage since surface stubble residue promotes cool, moist microhabitats (Dosdall et al. 1999). In conjunction with the above control measures, an integrated pest management plan can be devised to manage flea beetle populations and provide the lowest amount of environmental impact as possible.

1.2.2 Interactions of Flea Beetles with Volatilve Secondary Compounds

Under olfactometer experimentation, allyl-isothiocyanate acted as an attractant to spring-emerged beetles while conversely acting as a repellent to the offspring generation during the fall season (Gruber et al. 2009). This activity demonstrates a complex interaction of chemical signals responded to by flea beetles. Flea beetle feeding stimuli include glucosinolates, the volatile isothiocyanate and nitrile degradation products (Feeney et al. 1970; Hicks 1974). Since the later, fall generation may have been preparing for diapause under leaf litter and within shelter belts, the allyl-isothiocyanate compounds may help them determine suitable and/or unsuitable overwintering sites by directing them away from their food source (Gruber et al. 2009). Nitriles seemed to have a very weak influence on flea beetle response when compared to *Brassica*-produced terpenes found universally amongst other plant species. Flea beetles have also demonstrated activity influenced by green leaf compounds; however, not significantly so. It

is believed that the chemical signal perceived from crucifer-specific isothiocyanates and green leaf compounds affect flea beetle activity and retain their crucifer-specificity (Gruber et al. 2009). Six terpenes, (+)-linalool, (+)-limonene, (1*S*)-β- pinene, geraniol, β-ionone, (-)-*E*-caryophyllene, and indole demonstrated high inhibitory responses from *Phyllotreta* spp. yet had varying effects on other insects (Gruber et al. 2009). There are many known and unknown compounds produced by Brassicaceae with known and unknown functions. Identification and study of these compounds may lead to the eventual production of crop varieties with a high resistance to flea beetle attack, a strategy which may also be applied to other plant pests and pathogens.

1.3 Host Plant Resistance, PR Proteins and Cytokinins

Cultural control practices are important for minimizing damage from flea beetle attack. However, on its own, cultural control is rarely sufficient to reduce pest populations below damage thresholds (Dent 2000). Cultural practices can be even more effective if integrated with adoption of tolerant or resistant germplasm (Dent 2000).

Several researchers have investigated resistance in canola germplasm as a possible mechanism for reducing field infestations of these pests and consequently insecticide applications in agroecosystems. Early research by Lamb (1980, 1984, 1988a), Lamb and Palaniswamy (1990), and Bodnaryk and Lamb (1991) determined that species of Brassicaceae and cultivars within species differed in their susceptibilities to attack by flea beetles. Seedlings of white mustard, *S. alba*, and pennycress, *Thlaspi arvense* L., were found to be quite resistant to these pests (Bodnaryk and Lamb 1991; Meisner and Mitchell 1983). Leaves of *Brassica villosa* Biv. contain high trichome densities, making them highly resistant to flea beetle feeding (Palaniswamy and Bodnaryk 1994); the trichomes act as a physical barrier to flea beetles

preventing them from properly alighting on the leaf surface to initiate feeding. Gavloski et al. (2000) investigated several hybrids developed from intergeneric crosses of *S. alba* x *B. napus*, and found that two genotypes were particularly resistant to flea beetle attack. The authors concluded that resistance to flea beetles was a genetic trait found in some Brassicaceae that could be transferred by hybridization.

In addition to genetic crosses utilizing rather traditional breeding approaches to impart resistance to flea beetles, some research has also been conducted using transgenic strategies for moving genetic material to *B. napus* that confers resistance. Zaplachinski (1999) used a transgenic approach to express large quantities of proteinase inhibitors in canola seedlings during the first 5 to 10 days after germination. Specifically, the rice cysteine proteinase inhibitor, oxyzacystatin I, was expressed in transgenic *B. napus* as a fusion with an 18 kDa seed oleosin. The fusion site between the oleosin and the oryzacystatin contained a recognition sequence for collaginase derived from a marine bacterium. To help ensure that the proteinase inhibitor was expressed in the seedlings, the plants most expressing oleosin-oryzacystatin were crossed to another transgenic line containing the collagenase gene under control of a germination-specific promoter. Zaplachinski (1999) demonstrated that the transgenic lines containing cystatin were more resistant to flea beetle attack than controls, and that the transgenic lines had a negative effect on survival of adult flea beetles.

In a subsequent transgenic approach, Gruber et al. (2006) transformed *B. napus* with an Arabidopsis gene that produced plants with a dense covering of trichomes on stems and young leaves of seedlings. In subsequent field and laboratory studies, Soroka et al. (2011) evaluated trichome-enhanced transgenic canola seedlings for resistance to attack by flea beetles and found decreased flea beetle preference for the transgenic plants. The beetles demonstrated

altered behavior and lowered feeding activity on the trichome-enhanced seedlings relative to control seedlings with fewer trichomes.

Recent research has revealed an increase in abundance of pathogenesis-related (PR) proteins of the PR10 family in the presence of pathogens or abiotic stress. Originally identified in parsley (*Petroselinum* crispum (Mill.) Fuss.) (Somssich et al. 1988), they have been isolated in both mono- and dicotyledonous plants (Maleck et al. 2000; Srivastava et al. 2004; Liu and Xue 2006; Zubini et al. 2009; Zhijian et al. 2011). PR proteins are classified under 17 different families based on structure and function, many of which have yet to be defined (Zubini et al. 2009). Containing roughly 155 to 163 residues, the cytoplasmic PR10 proteins with an acidic pH demonstrate high variability in their primary structures. Nuclear Magnetic Resonance (NMR) and x-ray crystallography have demonstrated that PR proteins' three-dimensional folded structures are highly conserved with a seven-stranded β-sheet encircling two small α-helices and a C-terminal α-helix (Markovic-Housely et al. 2003; Liu et al. 2003; Srivastava et al. 2004; Chevalier et al. 2008; Zubini et al. 2009).

PR10 proteins are constitutively expressed throughout the plant's development in various tissues and organs. They are also upregulated during stress situations such as drought and/or pathogen attack (Kav et al. 2004; Liu et al. 2005; Srivastava et al. 2007; Chevalier et al. 2008; Zubini et al. 2009). Canola expressing a PR10 cDNA (ABR17; PR10.4), has shown faster germination and seedling development compared to non-transgenic controls (Srivastava et al. 2004). The ability of PR10 proteins to hydrolyze RNA seems to be linked with their role in plant stress response. RNA hydrolysis may function to degrade foreign pathogenic RNA while controlling its proliferation through infected cells by activating apoptosis (Zubini et al. 2009). Besides possessing RNase activity, its specific role in plant development is currently unknown since it is expressed during normal and stressful growth (Liu et al. 2003).

Cytokinins are an important class of phytohormones and have been shown to function as signal elements regulating plant growth and development. They have been credited with regulating leaf senescence and ensuring reproductive competence, and are found within regions of high metabolic activity in plants. They are found predominantly in plant meristems and are necessary for cell division (Matsumoto-Kitano et al. 2008; Nieminen et al. 2008). Natural cytokinins are N^6-substituted adenine derivatives that typically contain an isoprene-derivative side chain (Haber and Kieber 2002; Hwang and Sakakibara 2005; Rashotte et al. 2003). Cytokinin side chains vary by the presence or absence of an hydroxyl group at the terminal and stereo-isomeric position (Hwang and Sakakibara 2005). Synthetic cytokinins, on the other hand, are derived from diphenylurea and differ structurally from adenine-based cytokinins (Haberer and Kieber 2002).

The precise function(s) of cytokinin is still under investigation; however, in conjunction with their previously studied roles, elevated cytokinin concentrations are associated with cellular apoptosis and increased PR10 protein expression (Carimi et al. 2003; Zubini et al. 2009; Srivastava et al. 2007; Chevalier et al. 2008). PR10 proteins have been shown to interact with cytokinins, brassinosteroids and fatty acids (Srivastava et al. 2007; Chevalier et al. 2008; Fernandes et al. 2008). PR proteins have proven useful with respect to the plant's response to abiotic stress. *Arabidopsis thaliana* mutants constitutively expressing the *Pisum sativum* L. ABR-17 of the PR10 family associated with salt stress produced cytokinin concentrations three times higher than the wild type control (Srivastava et al. 2007; Krishnaswamy et al. 2008). This suggests a definite relationship between cytokinins and PR10 proteins with respect to functionality.

1.3.1 Protein Expression and Identification

Under normal growth and development or stress conditions, whether biotic or abiotic, a plant's proteome will change accordingly. Certain proteins may be constitutively expressed throughout varying stressful cycles; however, different proteins with different functions emerge as well. For instance, the fungal pathogen sclerotinia stem rot, *Sclerotinia sclerotiorum* (Lib.) de Bary, causes significant yield losses to canola crops. Using techniques such as 2-dimensional gel electrophoresis (2DGE), mass spectrometry and quantitative real-time polymerase chain reactions (qRT-PCR) can serve to elucidate proteome level changes between control, uninfected plants and infected plants (Liang et al. 2008). Identifying these proteins can then lead future breeding programs in directions not otherwise available.

Sclerotinia stem rot typically occurs near the end of flowering, in which the airborne ascospores infect the plant's flowers. Infected petals die and fall on leaves and leaf axils propagating the infection through the stem, causing plant death (Liang et al. 2008). An initial study based on proteome-level changes in the host plant caused by sclerotinia observed that 32 proteins were expressed by the host in response to infection. Two-dimensional gel electrophoresis (2-DGE) and mass spectometry (MS) were used to identify the sequence of these proteins.

The first dimension of 2-DGE entails separation of proteins based on their charge using isoelectric focusing (IEF). The proteins are rehydrated onto an IEF strip, a charge is applied causing the proteins to migrate the length of the strip until they reach their isoelectric point. The IEF strip is then added to the second dimension, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), at which point the proteins are passed through a gel as a charge is applied to cause their separation based on size. The pore size of the gel can be modified to allow for larger or smaller proteins to pass through. After staining, the gels can be scanned and run

through an analysis program so changes in the proteome can be noted for mass spectrometry analysis.

Images of the 2-dimensional (2-D) gels were acquired using a GS-800 calibrated densitometer (Bio-Rad) and analyzed with PDQuest 2-D analysis software using the Student's *t*-test feature at a significance level of $P < 0.05$ (version 7.3.1, Bio-Rad) as described in Liang et al. (2008). Quantities of statistically significant spots were determined using the spot quantification tool and fold-changes calculated (Liang et al. 2008). Of the statistically significant spots obtained, only those showing reproducible changes (up- or down-regulation) in all replicates were selected to undergo LC/MS/MS analysis at the Institute for Biomolecular Design (University of Alberta, Edmonton, Alberta, Canada).

1.3.2 Insect Resistance Management

If transgenic canola that expresses PR proteins is effective for reducing infestations of flea beetles, maintaining the resistance trait will be a crucial element when utilizing the germplasm in agroecosystems. Misuse of genetically modified crops and subsequent development of resistance by field populations of insects is a serious concern. Insect resistance management was conceived a decade prior to the release of crops engineered to express the toxin produced by the bacterium, *Bacillus thuringiensis* Berliner (*Bt*), with the goal of delaying field-evolved resistance (Bates et al. 2005). Several insect resistance management techniques are employed to help increase the longevity of the genetically modified crop and its novel gene. Using *Bt*-crops expressing moderate toxin dosages helps to manage pest outbreaks while allowing susceptible members of the pest population to survive and proliferate. One issue that arises from reduced dosage is that the toxin may have variable success at lower levels. Also,

models have shown that although reduced dosages function as described, they do not greatly extend the longevity of the novel gene (Bates et al. 2005; MacIntosh 2009).

In contrast, *Bt*-crops expressing high toxin dosages are utilized to kill insects possessing heterozygous genes for resistance (Bates et al. 2005; MacIntosh 2009). Since individuals with homozygous genes are expected to occur at extremely low rates, resistance evolution is propelled largely by the survival of individuals with heterozygote genes. Therefore, employing a high dose toxin will effectively eliminate heterozygotes from the population and greatly extend the longevity of the gene. This method seems to work only if it used sparingly. Regularly growing crops expressing high levels of the toxin would only increase the rate of resistance by selecting for resistant populations within the overall pest population (Bates et al. 2005; MacIntosh 2009).

Another insect resistance management technique involves the pyramiding of toxins. This method utilizes the expression of two or more toxins within a single variety. The idea behind pyramiding is that by using two different toxins with (ideally) two different binding sites, it is much more difficult to select for a population resistant to both toxins than for one (Bates et al. 2005). This strategy seems to be garnering the most support. However, as with any other method of control, moderation is vital. Although it will be more difficult to derive a population resistant to both toxins, this is still possible, and high selection pressure due to pyramiding can eventually result in development of resistant populations if not managed correctly.

Refuge zones allow for a mixture of *Bt*- and non-*Bt*-crops within the same field. The idea is to preserve susceptible pest populations through random mating of resistant and non-resistant populations (Bates et al. 2005). There is a high degree of difficulty involved with this strategy since it requires the maintenance of refuge zones and the susceptible population. Larval death due to inter-feeding on *Bt*- and non-*Bt*-crops reduces the susceptible population.

Since larvae are active and move throughout the field their possible exposure to *Bt*-crops increases.

1.4 Research Goal and Objectives

The overall goal of my thesis research was to investigate new strategies for reducing the susceptibility of canola to attack by flea beetles. At present, flea beetles are the most economically damaging insect pests in canola production (Madder and Stemeroff 1988). The vast insecticide quantities that are applied to treat seed prior to planting results in enormous cost to the canola industry both economically and environmentally. If successful, new control strategies could substantially enhance the sustainability of canola production.

Within this goal, I had several more specific research objectives. My research investigated new canola germplasm, transgenically modified to express the PR10 gene, for susceptibility to attack by flea beetles. My hypothesis was that since these transgenic lines produce greater quantities of cytokinins and consequently show more rapid seedling growth, the novel germplasm will have greater tolerance of feeding by adult flea beetles. A further objective was to investigate canola seed treatment with cytokinin for enhancing seedling growth and so fostering improved tolerance of seedlings to flea beetle attack. Since increased cytokinin concentrations have been linked to increased seedling vigor (Srivastava et al. 2007) and pathogenesis-related protein expression (Zubini et al. 2009), I hypothesized that cytokinin imbibition will confer increased resistance to flea beetle feeding.

A final objective was to compare changes in protein composition between seedlings of transgenic canola that have been fed upon by flea beetles with seedlings that have not been fed upon**.** My hypothesis is that since *B. napus* mutants expressing pea ABR17 exhibit enhanced

early seedling growth (Srivastava et al. 2007), it may also possess antixenotic properties. Mutants expressing pea ABR17 have yet to be tested against insect pests and this study is the first of its kind.

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Chapter 2. The Effectiveness of Pathogenesis-related Proteins and Cytokinins for Increasing the Tolerance of Seedlings of Canola, *Brassica napus* L., to Attack by Flea Beetles (Coleoptera: Chrysomelidae)

2.1 Introduction

Flea beetles (*Phyllotreta* spp.) (Coleoptera: Chrysomelidae) are major pests of the Brassicaceae in North America, Europe, and Asia (Lamb 1989). For instance, an American survey of New Jersey, New York and Pennsylvania found that 68% of organic broccoli producers ranked flea beetles among their top three crop pests while 27% of conventional farmers agreed (Anderssen et al. 2006; Hoffman et al. 1997). In North America, *Phyllotreta cruciferae* (Goeze) and *Phyllotreta striolata* (Fabricius) are among the dominant pests in canola (*Brassica napus* L.) (Madder and Stemeroff 1988; Philip and Mengersen 1989). In Canada alone, canola accounts for an annual revenue of approximately $15.4 billion (Canola Council of Canada 2011). Introduced from Eurasia, flea beetles have spread across temperate climates in the northern United States and southern Canada (Smith 1973). Flea beetles are responsible for reduced crop stands and delayed maturity leading to lower seed quantity and quality (Westdal and Romanow 1972).

Adults of *Phyllotreta* spp. overwinter under stubble and leaf litter in mid- to late September and emerge in mid- to late May. They are sustained on volunteer canola or brassicaceous weeds before moving into commercial fields to attack the spring-planted seedlings (Burgess 1977; Ulmer and Dosdall 2006; Gruber et al. 2009). Adults feed throughout the growing season with most of their damage restricted to the cotyledons in the early seedling phase. Once past this vulnerable growth stage, the plants are much more resistant to feeding damage (Gavloski and Lamb 2000). Their generational turnover is dependent on the climate and

host plant health but is typically restricted to two generations, one in the spring and one in late summer. The new generation of emerging adults feed on the foliage and siliques of mature canola plants, sometimes causing reduced seed quality and production (Knodel and Olson 2002).

Research into pathogenesis-related (PR) proteins of the PR10 family has revealed that their expression can be both constitutive and induced. Stress conditions such as drought or pathogen attack can result in their upregulation (Kav et al. 2004; Liu et al. 2005; Srivastava et al. 2007; Chevalier et al. 2008; Zubini et al. 2009). Expression of the PR10 cDNA (PR10.4 or ABR17) has resulted in seedlings displaying faster germination and early seedling vigour when compared with non-transgenic lines (Kav et al. 2004; Srivastava et al. 2004; Srivastava et al. 2006). Cytokinins, an important class of phytohormones, are known to be involved in signaling as well as plant growth and development, and have also been shown to increase in abundance in response to PR10 expression (Srivastava et al. 2007). The importance of PR10 proteins and cytokinins in the enhancement of seedling vigor prompted this study to investigate their effectiveness for increasing the tolerance of canola seedlings expressing PR10 (ABR17; PR10.1) to attack by flea beetles.

The objectives of this study were to: (1) test the ability of ABR17 (PR10.4) –and PR10.1-transgenic canola to withstand attack by both flea beetle species; (2) compare tolerance achieved with the transgenic canola germplasm with that achieved with insecticidal seed treatments; and (3) to determine the effect of cytokinin treatment on the ability of canola seedlings to tolerate and compensate for flea beetle attack.

2.2 Materials and Methods

2.2.1 Plants and Seed Treatments

Seedlings were grown on a greenhouse bench at the University of Alberta, Edmonton in 7-dram (25.88 mL) plastic cylindrical vials, with perforated bottoms to allow for water uptake from a tray in which they were held. The vials were held in groups of 10, all subjected to the same treatment, and potted with commercial potting mix, Sunshine Professional®, Sungro Horticulture. Sunshine Professional® contained fertilizer, Sphagnum peat moss, a wetting agent and lime. The seeds were sewn individually in the vials and watered daily over the course of 7 days.

Prior to experimental treatments, all seeds underwent surface sterilization and seed splitting procedures to ensure uniformity upon seeding. Surface sterilization of the seeds was conducted to remove the waxy outer layer of the seed coat to increase the uptake of 6-benzylaminopurine (BAP). Seeds of the *B. napus* doubled haploid (DH), Westar, PR10.1 or PR10.4 lines were counted in batches of 100 and placed in 1.5 mL tubes. To remove the waxy surface layer, 70% ethanol was applied and the seeds incubated for 20 minutes. After this time the ethanol was removed with a pipette and washed three times, 1 minute each, with autoclaved double distilled H_2O (ddH_2O). To sterilize the seed surface, 1 mL of 20% bleach was applied to the seeds for 10 minutes again with autoclaved ddH_2O as indicated earlier. Autoclaved ddH_2O was added to the seeds and they were incubated at room temperature for 24 hours to promote seed splitting. After 24 hours, the autoclaved ddH_2O was removed and replaced with concentrations of BAP of 0, 5, 25, 50 and 100 µM for three hours. Seeds were then washed three times with autoclaved ddH_2O and used for seeding.

The experimental treatments included canola seed of several types: (1) two *B. napus* controls, comprising a doubled haploid and *B. napus* cv. Westar with no insecticide or cytokinin

(6-benzylaminopurine) treatment; (2) two transgenic genotypes derived from the controls and comprised of ABR17 derived from doubled haploid lines and PR10.1 derived from Westar; (3) doubled haploid and Westar seeds imbibed in 5 µM BAP; (4) doubled haploid and Westar seeds imbibed in 25 µM BAP; (5) doubled haploid and Westar seeds imbibed in 50 µM BAP; (6) doubled haploid and Westar seed imbibed in 100 µM BAP; and (7) seeds of the doubled haploid and Westar controls coated with the neonicotinoid insecticide, thiamethoxam.

Seeds subjected to treatment with insecticide (Helix® or thiamethoxam) were surface sterilized as described above and then placed in a Petri dish and coated with concentrate (containing 10.3% thiamethoxam, 1.24% difenoconazole, 0.39% metalaxyl-M, and 0.135% fludioxonil) at the manufacturer's recommended rate.

2.2.2 Trial Design and Damage Assessments

The study involved three distinct experiments. In Experiment 1, the susceptibility to attack by *P. cruciferae* and *P. striolata* was evaluated for seedlings of the transgenic genotype, ABR17-3.15, compared with the doubled haploid control and the doubled haploid seeds subjected to treatment with thiamethoxam. In Experiment 2, the susceptibility to attack by *P. cruciferae* and *P. striolata* was evaluated for seedlings of the transgenic genotype PR10.1 compared with the Westar control and Westar seeds subjected to treatment with thiamethoxam. In Experiment 3, the susceptibility to attack by *P. cruciferae* and *P. striolata* was evaluated for seedlings of doubled haploid and Westar that were imbibed in solutions of 0, 5, 25, 50, and 100 µM BAP. For each experiment, trials were conducted with densities of two and four adult beetles per seedling.

Trials were conducted according to the method of Tansey et al. (2008). Beetle specimens were collected from canola fields and in patches of brassicaceous weeds near

Edmonton, AB, Canada. Adults of the overwintered and summer/fall generation were collected from May to June and August to September 2009 and 2010 respectively, using insect sweep nets and hand collections with battery-powered aspirators. Beetles were returned to the laboratory and held in insect cages (40.5 by 40.5 cm at the base and 80.5 cm in height, lined on the sides with 500-µm Nitex mesh screening) containing potted plants of *B. napus* cv. Q2. After seven days the most robust seedlings with fully expanded cotyledons were selected for the experiments. Sixteen seedlings were placed in a 4 x 4 formation in the centre of a 60 x 60 x 90 mesh cage with no space between the adjacent plants. Beetles were introduced to the caged seedlings within one week of capture. Each experiment was conducted with every seedling type with four replications.

The trials were conducted for 72 hours, after which the flea beetles were removed, mortality recorded, and seedlings scored on a defoliation scale of 0-10 similar to that proposed by Palaniswamy et al. (1992). A rating of 0 corresponded to 0% defoliation, 1 with 1-10% and 10 with 91-100% defoliation. Experiments were performed in University of Alberta, Edmonton laboratories with ambient light and a photoperiod of ca. 16 hours light : 8 hours dark in mid-May to mid-September.

2.2.3 Data Analysis

Corrected mortality values were calculated using the Schneider-Orelli (1947) formula:

$$M(\%) = [(t-c)/(100-c)] \times 100$$

M(%) is the relative mortality, *c* represents the average percentage mortality in the controls and *t* represents the percentage mortality in the treatment groups. Feeding damage ratings and corrected mortalities were compared using analysis of variance (ANOVA) of the

randomized complete block design using SAS version 9.1 (SAS Institute 2005) as per Tansey et al. (2008).

2.3 Results

Observed flea beetle feeding was typical of damage reported previously by other researchers (e.g., Burgess 1977; Westdal and Romanow 1972; Philip and Mengersen 1989; Tansey et al. 2008). Damage appeared as small holes chewed out of the cotyledons with occasional stem severance. A number of control and treated plants, including the transgenic lines, had cotyledons partially or completely destroyed by both species after the 72 h feeding period. No obvious feeding pattern was observed, and damage was indistinguishable between the two *Phyllotreta* species.

2.3.1 Feeding damage

2.3.1.1 Flea Beetle Feeding Damage to the Transgenic Genotype ABR17-3.15, Its Doubled Haploid wild type Counterpart, and Seedlings Subjected to an Insecticide Treatment (Experiment 1)

Flea beetle density differences of two versus four specimens of *Phyllotreta* spp. per plant significantly affected feeding damage ($F = 19.34$; d.f. = 1, 38; $P < 0.0001$). Significant differences were observed among treatments ($F = 33.52$; d.f. = 2, 38; $P < 0.0001$). *Phyllotreta striolata* caused significantly more damage to seedlings than *P. cruciferae* ($F = 37.46$; d.f. = 1, 38; $P < 0.0001$). A significant density by treatment interaction was observed ($F = 3.54$; d.f. = 2, 38; $P = 0.0391$), as well as a treatment by beetle species interaction ($F = 16.86$; d.f. = 2, 38; $P < 0.0001$). However, the density by treatment by flea beetle species interaction was not significant ($F = 2.67$; d.f. = 2, 38; $P = 0.082$).

At a density of two *P. cruciferae* per plant, *ABR17*-3.15 and the Helix®-treated doubled haploid displayed significantly lower least squares (LS) mean feeding damage than the doubled haploid wild type control ($P < 0.05$) (Fig. 1). There was no significant difference in mean feeding damage between *ABR17*-3.15 and the Helix®-treated doubled haploid. At a density of two *P. striolata* per plant, *ABR17*-3.15 demonstrated significantly greater feeding damage than the Helix®-treated doubled haploid (Fig. 1). *ABR17*-3.15 had significantly greater feeding damage at a density of two *P. striolata* per plant than for the same density of *P. cruciferae*. Feeding damage to the doubled haploid control at a density of two *P. striolata* per plant was similar to that of *ABR17*-3.15 seedlings. The doubled haploid control at two *P. cruciferae* per plant showed no statistically significant difference when compared with the doubled haploid control at two *P. striolata* per plant. The Helix®-treated doubled haploid treatment showed no significant difference in feeding damage for the two beetle species at a density of two beetles per plant (Fig. 1).

At a density of four *P. cruciferae* per plant, no significant differences were observed for feeding damage between the transgenic *ABR17*-3.15 and its doubled haploid control, or between *ABR17*-3.15 and the Helix®-treated doubled haploid seedlings (Fig. 2). However, at a density of four *P. cruciferae* per seedling, feeding damage to the doubled haploid control significantly exceeded that of the Helix®-treated doubled haploid seedlings. At four *P. striolata* per plant, the Helix®-treated doubled haploid had significantly less feeding damage than either *ABR17*-3.15 or the doubled haploid control seedlings. There were no significant differences in feeding damage between the *ABR17*-3.15 and the doubled haploid control. Both the *ABR17*-3.15 genotype and the doubled haploid control had significantly reduced LS mean feeding damage at four *P. cruciferae* per plant versus the same density of *P. striolata*. There was no significant

difference between the Helix®-treated doubled haploid at four *P. striolata* per plant when compared with the same treatment and density of *P. cruciferae* (Fig. 2).

2.3.1.2 Flea Beetle Feeding Damage to the Transgenic Genotype Expressing PR10.1 cDNA, B. napus cv. Westar, and Seedlings Subjected to an Insecticide Treatment (Experiment 2)

A significant difference in feeding damage was observed between the two and four flea beetle per plant densities ($F = 21.09$; d.f. = 1, 43; $P < 0.0001$). Significant differences were also observed among treatments ($F = 29.21$; d.f. = 2, 43; $P < 0.0001$) and between beetle species ($F = 9.88$; d.f. = 1, 43; $P = 0.003$). No significant interactions were observed between treatment and beetle species ($F = 3.00$; d.f. = 2, 43; $P = 0.601$), or among beetle density, treatment and beetle species ($F = 0.55$; d.f. = 2, 43; $P = 0.5836$)

No significant difference in feeding damage was observed when comparing the transgenic genotype expressing *PR10.1* cDNA with the Westar control treatment at two *P. cruciferae* per plant (Fig. 3). However, significantly greater feeding damage was observed to Westar control seedlings compared to seedlings of Helix®-treated Westar. With a density of two *P. striolata* per plant, no statistically significant differences were observed when comparing Helix®-treated Westar and PR10.1. Both PR10.1 and the Westar control had significantly more feeding damage at two *P. striolata* per plant than with two *P. cruciferae* per plant (Fig. 3).

The Westar control treatment at four *P. cruciferae* per plant demonstrated significantly greater feeding damage than PR10.1 and Helix®-treated Westar (Fig. 4). However, PR10.1 and Helix®-treated Westar showed no significant differences when compared with each other at four *P. cruciferae* per plant. Helix®-treated Westar had similar feeding damage to PR10.1 at a density of four *P. striolata* per plant. No significant differences in feeding damage were

observed between PR10.1 and the Westar control at four *P. striolata* per seedling, but feeding damage to the Westar control significantly exceeded that to Helix®-treated Westar. There were no statistically significant differences in treatment effects between beetle species at the density of four beetles per plant (Fig. 4).

2.3.1.3 Flea Beetle Feeding Damage to B. napus Doubled Haploid and cv. Westar Following Seed Treatments with Cytokinin (Experiment 3)

Beetle density significantly affected feeding damage to seedlings (F = 64.024; d.f. = 1, 117; P < 0.0001). Treatment effects (F = 2.72; d.f. = 7, 117; P = 0.012) and beetle species (F = 84.81; d.f. = 1, 117; P = <0.0001) also demonstrated significant effects on feeding damage. Similarly, significant effects were observed for the density by treatment interaction (F = 2.81; d.f. = 7, 117; P = 0.0096), as well as the density by treatment by beetle species interaction (F = 3.17; d.f. = 7, 117; P = 0.0042). Meanwhile, no significant differences were observed for the density by beetle species interaction (F = 0.85; d.f. = 1, 117; P = 0.3588) or the treatment by beetle species interaction (F = 0.79; d.f. = 7, 117; P = 0.5979).

Seedlings of *B. napus* doubled haploids subjected to seed imbibition with 25 µM of BAP exhibited significantly lower LS mean damage at two *P. cruciferae* per plant than all other treatments except seedlings that developed from seed treated with 50 µM of BAP (Fig. 5). Doubled haploid seedlings treated with 25 µM of BAP also had significantly less mean feeding damage than doubled haploids treated with 5 µM BAP at the same density and beetle species. Doubled haploid seedlings treated with 5 µM BAP and a density of two *P. striolata* per plant had numerically the highest LS mean damage, although it was not significantly different from damage by *P. striolata* to doubled haploid seedlings treated with higher application rates of BAP. Seedlings of *B. napus* cv. Westar treated with 25 µM BAP and a density of two *P. striolata*

per plant had the lowest LS mean damage among the *P. striolata* treatments, and damage to these seedlings was significantly less than that to Westar seedlings treated with 50 and 100 µM BAP, and to doubled haploids treated with 5, 25, and 100 µM BAP. No statistically significant differences in feeding damage were observed for doubled haploid seedlings subjected to attack by two *P. cruciferae* per plant and treated with 5 and 100 µM BAP or to seedlings of Westar treated with 50 and 100 µM BAP (Fig. 5).

For seedlings subjected to attack by two *P. cruciferae* per plant, a rate effect was evident for seedlings of Westar in which LS mean damage gradually increased with BAP application rate from 5 to 100 µM; a similar rate effect was evident for doubled haploid seedlings with BAP application rates ranging from 25 to 100 µM (Fig. 5). However, a similar BAP rate effect on flea beetle feeding damage was not evident for seedlings subjected to attack by two *P. striolata* per plant (Fig. 5).

At the density of four beetles per plant, results for the BAP-treated *B. napus* were more uniform than at two beetles per plant. Seedlings of Westar seed-treated with 25 µM BAP and a density of four *P. cruciferae* per plant had significantly less feeding damage than doubled haploid seedlings treated with 5 and 25 µM BAP, and Westar seedlings treated with 50 µM BAP at the same density and beetle species (Fig. 6). At four *P. striolata* per plant, Westar plants seed-treated with 25 µM BAP demonstrated the greatest LS mean damage, but this was significantly greater only from Westar plants subjected to 100 µM BAP, at the same density and beetle species. The remaining treatments at four *P. striolata* per plant demonstrated no significant LS mean damage values when compared with Westar 25 µM BAP and Westar 100 µM BAP. The only significant differences between the two *Phyllotreta* spp. was displayed between Westar 25 µM BAP at four *P. cruciferae* per plant, which had the lowest overall LS mean damage, and

Westar 25 µM BAP at four *P. striolata* per plant, which had the highest overall LS mean damage (Fig. 6).

2.3.2 Mortality

2.3.2.1 Flea Beetle Mortality for the Transgenic Genotype ABR17-3.15, Its Doubled Haploid Counterpart, and an Insecticide Treatment (Experiment 1)

No statistically significant differences in beetle mortality were observed in relation to beetle density of two and four specimens per plant (F = 2.2; d.f. = 1, 40; P = 0.1459), or beetle species (F = 0.17; d.f. = 1, 40; P = 0.6844) for LS means of corrected mortality. Treatment effects were significant (F = 4.79; d.f. = 2, 40; P = 0.0136), along with the density by beetle species interaction (F = 4.23; d.f. = 1, 40; P = 0.0463). The density by treatment interaction was not significant (F = 0.58; d.f. = 2, 40; P = 0.5666), nor was the treatment by beetle species interaction (F = 0.25; d.f. = 2, 40; P = 0.7788). Similarly, the density by treatment by beetle species interaction was nonsignificant (F = 1.06; d.f. = 2, 40; P = 0.3553).

The highest observed LS mean for corrected mortality at a density of two *Phyllotreta* spp. per plant was for *P. striolata* subjected to the doubled haploid Helix® treatment (Fig. 7). However, among all treatments, a statistically significant difference was only observed between the doubled haploid Helix® treatment at two *P. striolata* per plant and *ABR17*-3.15 at a density of two *P. cruciferae* per plant (Fig. 7).

At a density of four beetles per plant no significant differences in least squares mean mortality were observed among treatments for *P. cruciferae* (Fig. 8). However, significantly greater mortality occurred in the doubled haploid control seedlings than for *ABR17*-3.15 seedlings with *P. striolata*. Meanwhile, *ABR17*-3.15 and the doubled haploid control seedlings at four *P. cruciferae* per plant and the doubled haploid controls and Helix®-treated seedlings at

four *P. striolata* per plant showed no significant differences between each other in terms of beetle mortality (Fig. 8).

2.3.2.2 Flea Beetle Mortality for the Transgenic Genotype PR10.1, B. napus cv. Westar, and an Insecticide Treatment (Experiment 2)

Effects of beetle density (F = 0.38; d.f. = 1, 45; P = 0.5401) and species (F =0.00; d.f. = 1, 45; P = 0.9466) were nonsignificant in terms of the LS means of corrected mortality. Similarly, the density by treatment interaction (F = 0.66; d.f. = 2, 45; P = 0.524), the density by beetle species interaction (F = 1.02; d.f. = 1, 45; P = 0.3184) and the treatment by beetle species interaction (F = 0.58; d.f. = 2, 45; P = 0.5619) showed no significant effects. However, treatment effects were significant (F = 43.49; d.f. = 2, 45; P < 0.0001), but the density by treatment by beetle species interaction was nonsignificant (F = 0.31; d.f. = 2, 45; P = 0.7379).

Treatments with the highest LS means for corrected mortality, Helix®-treated Westar at two *P. cruciferae* and two *P. striolata* per plant, were not significantly different when compared with each other (Fig. 9). However, they did show statistically significant differences when compared against Westar controls at two *P. cruciferae* and two *P. striolata* per plant and their transgenic counterparts, PR10.1, at the same density for both species. Meanwhile, Westar controls at two *P. cruciferae* and two *P. striolata* per plant and PR10.1 at the same density showed no differences when compared with each other (Fig. 9).

Similar results were recorded at the higher beetle density. No statistically significant differences were observed between Helix®-treated Westar at four *P. cruciferae* and four *P. striolata* per plant (Fig. 10). Statistically significant differences were observed when Helix®-treated Westar was compared with Westar controls and PR10.1 at the same density for both

species. Analyzing Westar controls at four *P. cruciferae* and four *P. striolata* per plant and PR10.1 showed no significant differences (Fig. 10).

2.3.2.3 Flea Beetle Mortality for the Cytokinin treatments of B. napus Doubled Haploid and cv. Westar (Experiment 3)

Flea beetle mortality after feeding on seedlings subjected to various cytokinin applications was significantly affected by treatment ($F = 4.27$; d.f. = 7, 122; $P = 0.0003$), beetle species ($F = 13.41$; d.f. = 1, 122; $P = 0.0004$), and the density by treatment interaction ($F = 4.39$; d.f. = 7, 122; $P = 0.0002$). Similarly, the treatment by beetle species interaction ($F = 4.8$; d.f. = 7, 122; $P < 0.0001$) and the density by treatment by beetle species interaction ($F = 2.91$; d.f. = 7, 122; $P = 0.0076$) were significant. The only effects that showed no statistically significant differences were density ($F = 0.6$; d.f. = 1, 122; $P = 0.4403$) and the density by beetle interaction ($F = 1.98$; d.f. = 1, 122; $P = 0.1624$).

At a density of two *P. cruciferae* per plant, no significant differences in LS mean mortality levels were observed among seedlings of *B. napus* doubled haploids or their Westar counterparts when subjected to seed imbibitions of any of the BAP concentrations (Fig. 11). However, doubled haploids treated with 50 µM of BAP exhibited significantly higher LS mean corrected mortality when subjected to attack by *P. striolata* than for doubled haploid seedlings treated with 5, 25, 50 and 100 µM of BAP and fed upon by *P. cruciferae*. Westar treated with each of the BAP concentrations at a density of two *P. cruciferae* per plant displayed no significant differences in mortality when compared with all other treatments at two *Phyollotreta* spp. per plant (Fig. 11).

DH subjected to seed imbibitions of 5 µM of BAP at a density of four *P. striolata* per plant demonstrated the highest LS mean corrected mortality, and mortality significantly

exceeded that for all other treatments (Fig. 12). At the same beetle density and species, DH with 25 µM of BAP had the second highest LS mean mortality, and this significantly exceeded the corrected mortality from all other treatments (except doubled haploids seed-treated with 5 µM BAP). Mortality of *P. striolata* adults when feeding on Westar seedlings treated with 25 and 50 µm of BAP significantly exceeded that of *P. striolata* adults feeding on doubled haploids treated with 50 and 100 µM of BAP. Similarly, mortality of *P. cruciferae* adults feeding on Westar seedlings treated with 5, 25, 50, and 100 µm of BAP significantly exceeded that of *P. striolata* adults feeding on doubled haploids treated with 50 and 100 µm of BAP (Fig. 12).

2.4 Discussion

The most relevant discovery of this research was that both transgenic genotypes, *ABR17*-3.15 and PR10.1, caused reductions in feeding damage by *P. cruciferae* that were equivalent to their wild type counterparts seed-treated with thiamethoxam insecticide, regardless of whether densities were two or four beetles per plant. However, the same level of reduced damage was not observed for *P. striolata* at either of the densities evaluated. The results are quite relevant because *P. cruciferae* remains the dominant flea beetle species attacking canola in the Mixed Grassland, Moist Mixed Grassland, and Aspen Parkland Ecoregions of western North America (Dosdall and Mason 2010), an area encompassing many thousands of hectares of cropland. The greater feeding damage caused by *P. striolata* than *P. cruciferae* to seedling canola, and the stronger resistance of *P. striolata* to neonicotinoid insecticides have been reported previously (Tansey et al. 2008; Tansey et al. 2009), and this research has provided further evidence to support these differences among the congeneric species.

The mechanism of resistance by the *B. napus* genotype PR10.1 and *ABR17*-3.15 to *P. cruciferae* appears to involve nonpreference or antixenosis according to Painter (1951) and

Kogan and Ortman (1978), because increased mortality was not apparent on either PR10.1 and *ABR17*-3.15 (Figs. 7, 8, 9, 10). However, a combination of mechanisms that includes tolerance in addition to antixenosis also may be involved, but further study is needed to clarify this aspect.

My studies showed significant differences in relation to feeding damage when comparing the two beetle densities, regardless of species. At the highest beetle density of four per plant, greater feeding damage was observed than at the density of two adults per plant. This is to be expected as a greater beetle density, regardless of species of *Phyllotreta*, equates to less available plant material per individual. Flea beetle densities of two adults per seedling are relatively common throughout North American canola production regions and densities of four per plant can occur frequently (Hiiesaar et al. 2003; Tansey et al. 2008).

A variety of treatment effects were observed for both feeding damage and adult beetle mortality. Notably, Helix®-treated seedlings performed the best in terms of least plant damage and greatest beetle mortality under all density and beetle species treatments. Even when control and transgenic seedlings failed to perform equivalently at the same density for both beetle species, Helix®-treated seedlings provided the most consistent results. This is an important consideration because neonicotinoid insecticides, including Helix® or thiamethoxam, comprise the most common form of pesticide-based control for *Phyllotreta* spp. throughout North America (Dosdall 2009). Thiamethoxam is a second generation neonicotinoid belonging to the thianicotinyl chemistry subclass, and it acts through systemic action (Health Canada, Pest Management Regulatory Agency 2001). It is also the active ingredient in Helix Xtra® and Cruiser® seed treatments. Neonicotinoids are found in many broad-spectrum pesticides and act as cellular agonists for nicotinic acetylcholine receptors that are found in high densities within the insect nervous tissue (Brown et al. 2006).

Cytokinin treatments displayed an interesting trend as concentrations increased. At two *P. cruciferae* per plant, damage was significantly lower for doubled haploids treated with 25 than 5 µM of BAP, and damage increased between the 50 and 100 µM concentrations. *Brassica napus* cv. Westar displayed a numerical increase in feeding damage with increasing BAP concentrations; however, unlike the doubled haploid genotype, we observed no statistically significant effects and no substantial reduction in damage between 5 and 25 µM of BAP. A somewhat inverted but nonsignificant trend was observed with *B. napus* doubled haploids at a density of two *P. striolata* per plant as damage declined from 5 to 50 µM of BAP, but damage then increased for doubled haploids with 100 µM of BAP. Cytokinin-imbibed Westar showed a similar response for *P. striolata*: damage declined significantly from 5 to 25 µM of BAP, but then increased at concentrations of 50 and 100 µm BAP. At four beetles per plant, feeding damage was much more uniform and trends could not be discerned: the high density effect evidently resulted in extensive feeding damage so that less beetle selective capability was apparent. Several studies have analyzed cytokinins and their role in normal plant growth and development. Elevated endogenous levels of cytokinins have been observed with the upregulation of pathogenesis-related genes. Cytokinins can enhance germination and promote early seedling vigor under normal and abiotic stress conditions (Srivastava et al. 2007). Moreover, they can increase cambium development in root and shoot tissues (Matsumoto-Kitano et al. 2008; Nieminen et al. 2008), regulate leaf senescence and ensure reproductive competence (Sugawara et al. 2007). In conjunction with auxin phytohormones, cytokinins have been used to induce calli and regenerate roots and shoots (Vysotkaya et al. 2006; Neibaur et al. 2008). My hypothesis was that increasing levels of seed imbibition of cytokinins would promote seedling vigor, leading to less damage from flea beetle attack, but antixenotic properties may

exist at specific levels of BAP imbibition and so produce the variable results observed in this study.

The doubled haploid and Westar control treatments exhibited the highest damage ratings, as expected. Both the doubled haploid and Westar control treatments had significantly higher damage ratings than the Helix®-treated doubled haploid and Westar under all density and beetle species treatments. The doubled haploid controls only had significantly higher damage than the *ABR17*-3.15 transgenic at two *P. cruciferae* per plant. These results emphasize the high susceptibilities of the control treatments, and the potential role for transgenic genotype *ABR17*-3.15 in the integrated management of *P. cruciferae* in canola because damage inflicted to the transgenic line was not significantly different from that of thiamethoxam-treated seedlings.

Both transgenic genotypes, *ABR17*-3.15 and PR10.1, have not been previously tested for their responses to insect attack. Some previous studies have focused on their increased responses to abiotic stress tolerance; however, this study represents the first of its kind with respect to insect pest tolerance. These results are extremely promising and could have a positive impact on reducing environmental inputs of insecticides. Further research could validate whether or not *ABR17*-3.15 and PR10.1 could be used as a viable replacement for insecticide-treated seeds. Pathogenesis-related proteins have also proven useful with respect to the plant's response to abiotic stress, and this effect in combination with reduced insect attack could enhance usefulness of this trait in agroecosystems.

The most significant results obtained through mortality observations pertain to Helix®-treated seedlings. In both the doubled haploid and Westar trials, the Helix® treatments caused the greatest mortality to *Phyllotreta* spp. at a density of two per plant. At four *Phyllotreta* spp. per plant, Helix®-treated Westar caused significantly greater beetle mortality than all other

treatments. The transgenic genotypes do not appear to possess insecticidal properties comparable to Helix® treatments. Previous studies with flea beetles and neonicotinoid compounds reported an increased mortality rate of *P. cruciferae* over *P. striolata* as well as significantly greater mortality to both species when analyzed against the untreated control (Tansey et al. 2008; Tansey et al. 2009). Our results did not concur with their conclusions, a fact that could perhaps be attributed to somewhat different application methods of seed treatments in the two studies. Helix® applications for our study were performed in the laboratory in petri dishes with seed that had been surface sterilized, whereas seed applications in Tansey et al. (2008, 2009) were performed using commercial application technology. It is possible, therefore, that some differences occurred in the quantities of insecticide adsorbed onto the seed coats in the two studies. However, since the Helix®-treated Westar performed similarly to the results observed by Tansey et al. (2008, 2009) and the PR10.1 transgenic performed comparably against it, it is safe to assume that results are reproducible.

No dose response was observed for the cytokinin treatments and mortality: doubled haploids and Westar seedlings had similar effects on beetle mortality of *P. cruciferae* and *P. striolata* regardless of application rate. Interestingly, cytokinin treatments appeared to have increased numerical, but not statistically significant, mortality for two *P. striolata* per plant when compared against two *P. cruciferae* per plant. It is possible that cytokinin imbibition conveys some insecticidal effect at two *Phyllotreta* spp. per plant. Although significant research has been conducted on cytokinins and their role in plant growth and development, to my knowledge no previous work has studied cytokinins and their effect or response to insect attack.

In this study, we investigated both the overwintered, spring generation and newly emerged summer to fall generation of *P. cruciferae*. The second generation of *P. cruciferae* was found by Tansey et al. (2008, 2009) to be more tolerant to neonicotinoid insecticides, and it is

therefore possible that the two generations could respond differently to the transgenic genotypes and the cytokinin treatments investigated in this study. Future research could benefit from comparing responses of the two generations to factors investigated here.

At present, virtually all canola grown in North America is seed-treated with neonicotinoid insecticide to minimize crop losses from herbivory by flea beetles. Excessive reliance on these compounds over such a vast geographical area has placed enormous selection pressure on flea beetles to develop resistance to these compounds. Results of this study have shown exceptional promise for the *B. napus* transgenic genotypes, *ABR17*-3.15 and PR10.1, because they both imparted a level of antixenotic resistance to the principal flea beetle pest, *P. cruciferae*, that is comparable to that attained with the most commonly used insecticide, thiamethoxam. The introduction of these genotypes into canola cropping systems therefore has considerable promise for achieving more sustainable management of these pests. The most appropriate implementation of this new germplasm would be to integrate it with other cultural practices that have proven successful for reducing damage by these pests, such as increasing plant density (Dosdall et al. 1999; Dosdall and Stevenson 2005), growing canola in a zero tillage regime rather than with conventional tillage (Dosdall et al. 1999), and planting large rather than small seeds (Bodnaryk and Lamb 1991; Elliott et al. 2007), at wide row spacings (Dosdall et al. 1999). Results of this study therefore hold considerable promise for enabling canola producers to reduce their reliance on insecticide applications in canola production systems.

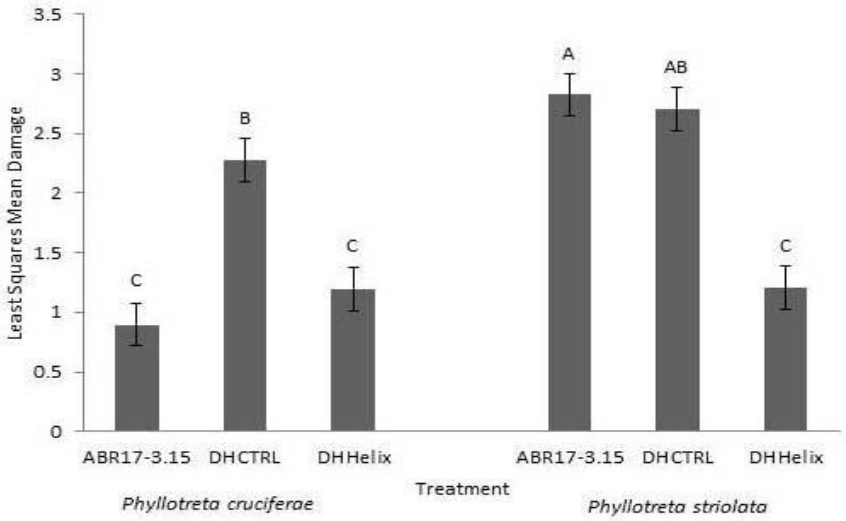

Figure 1. Least squares mean damage (± S.E.) to *Brassica napus* seedlings of the doubled haploid control (DHCTRL), the transgenic genotype (ABR17–3.15), and the doubled haploid control seed-treated with thiamethoxam insecticide (DHHelix) at a density of two adults of *Phyllotreta* spp. per plant. Letters on histograms indicate significance of differences among treatments: means with the same letter are not significantly different ($P > 0.05$).

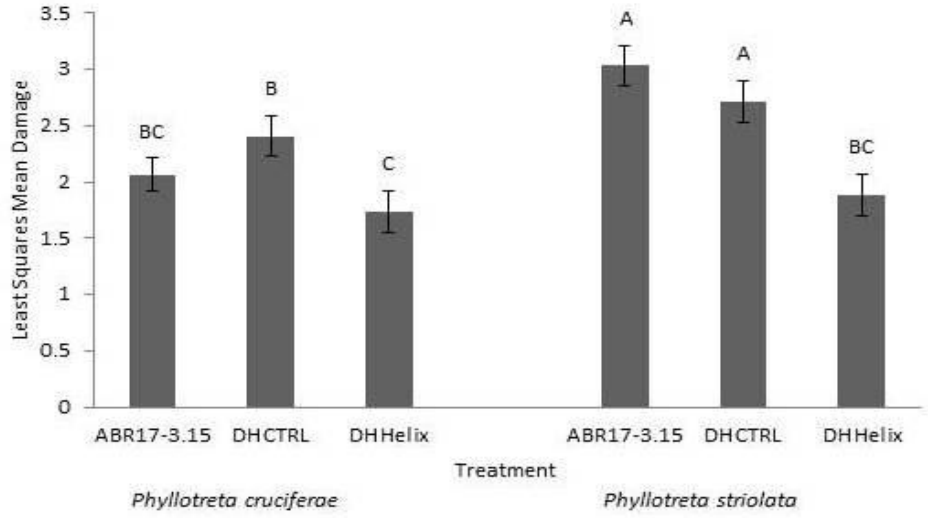

Figure 2. Least squares mean damage (± S.E.) to *Brassica napus* seedlings of the doubled haploid control (DHCTRL), the transgenic genotype (ABR17-3.15), and the doubled haploid control seed-treated with thiamethoxam insecticide (DHHelix) at a density of four adults of *Phyllotreta* spp. per plant. Letters on histograms indicate significance of differences among treatments: means with the same letter are not significantly different ($P > 0.05$).

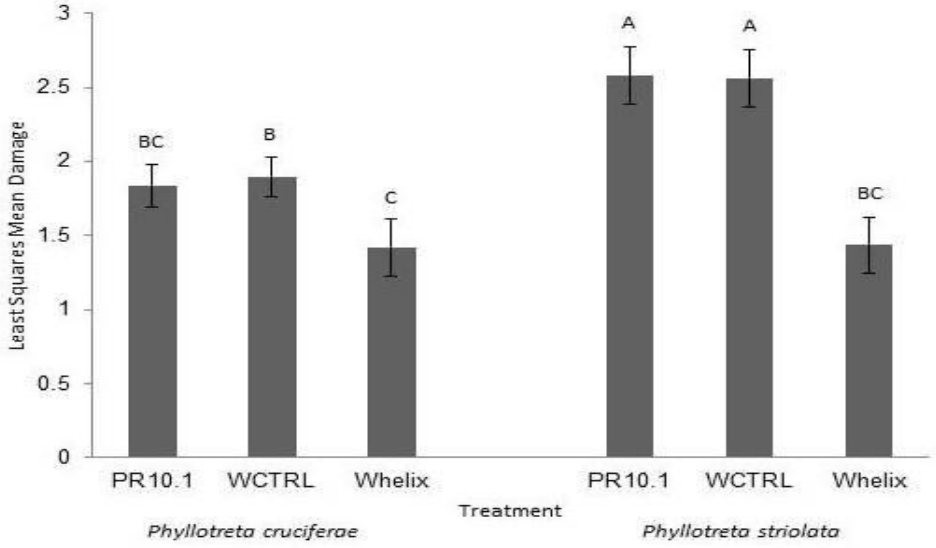

Figure 3. Least squares mean damage (± S.E.) to *Brassica napus* seedlings of the Westar control (WCTRL), its transgenic counterpart genotype (PR10.1), and Westar seed-treated with thiamethoxam insecticide (WHelix) at a density of two adults of *Phyllotreta* spp. per plant. Letters on histograms indicate significance of differences among treatments: means with the same letter are not significantly different ($P > 0.05$).

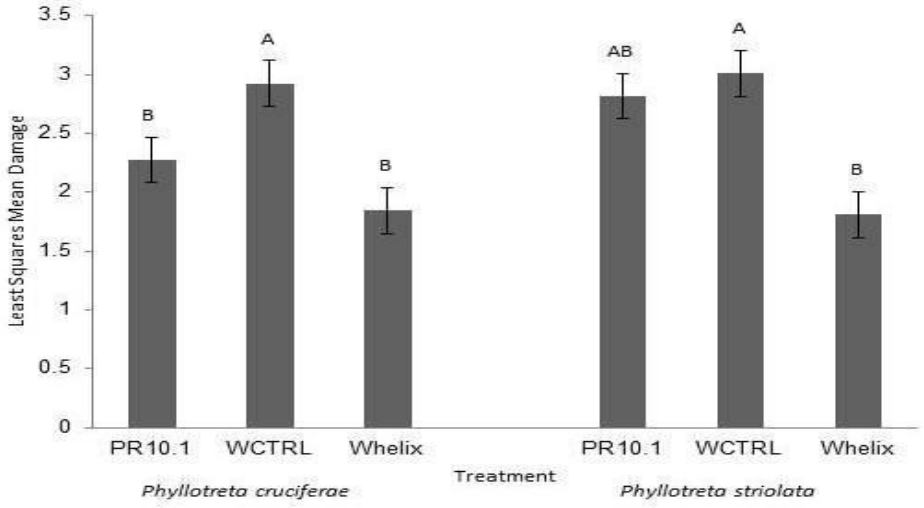

Figure 4. Least squares mean damage (± S.E.) to *Brassica napus* seedlings of the Westar control (WCTRL), its transgenic counterpart genotype (PR10.1), and Westar seed-treated with thiamethoxam insecticide (WHelix) at a density of four adults of *Phyllotreta* spp. per plant. Letters on histograms indicate significance of differences among treatments: means with the same letter are not significantly different ($P > 0.05$).

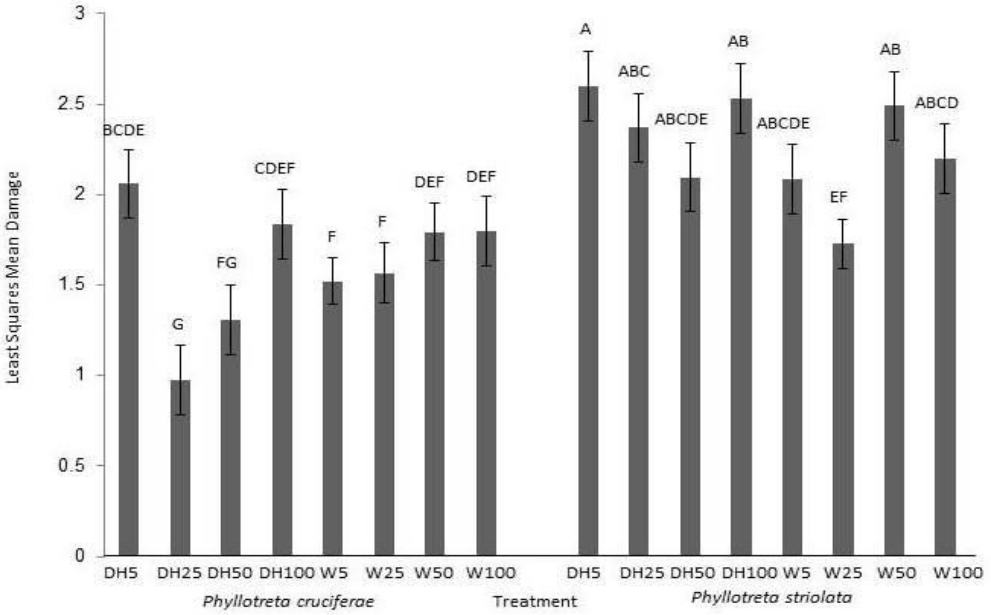

Figure 5. Least squares mean damage (± S.E.) to seedlings of *Brassica napus* cv. Westar (W) and doubled haploids (DH) seed-treated with 6-benzylaminopurine at application rates of 5, 25, 50, and 100 µM at a density of two adults of *Phyllotreta* spp. per plant. Letters on histograms indicate significance of differences among treatments: means with the same letter are not significantly different ($P > 0.05$).

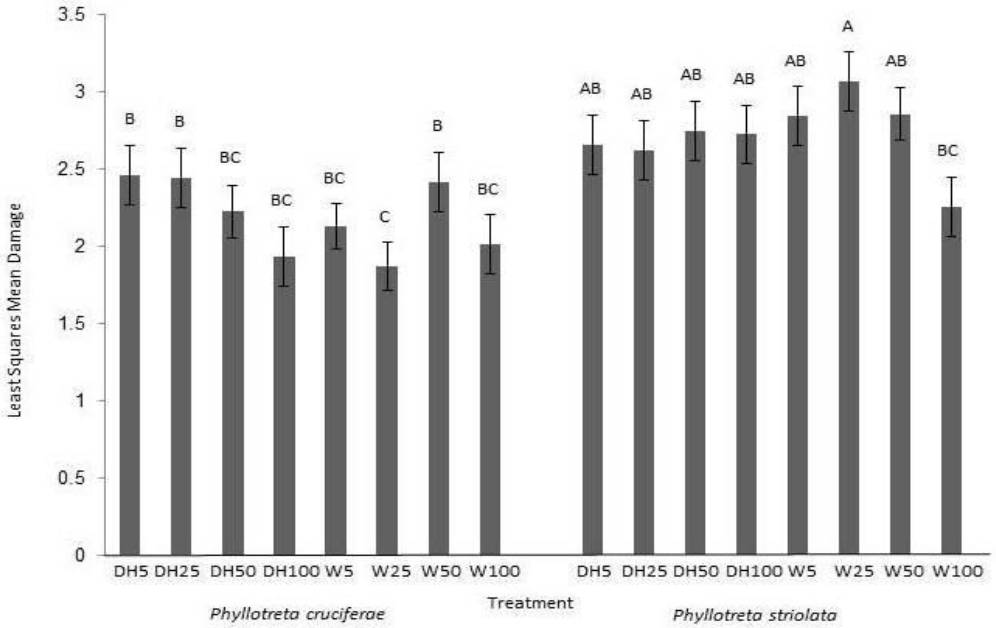

Figure 6. Least squares mean damage (± S.E.) to seedlings of *Brassica napus* cv. Westar (W) and doubled haploids (DH) seed-treated with 6-benzylaminopurine at application rates of 5, 25, 50, and 100 µM at a density of four adults of *Phyllotreta* spp. per plant. Letters on histograms indicate significance of differences among treatments: means with the same letter are not significantly different ($P > 0.05$).

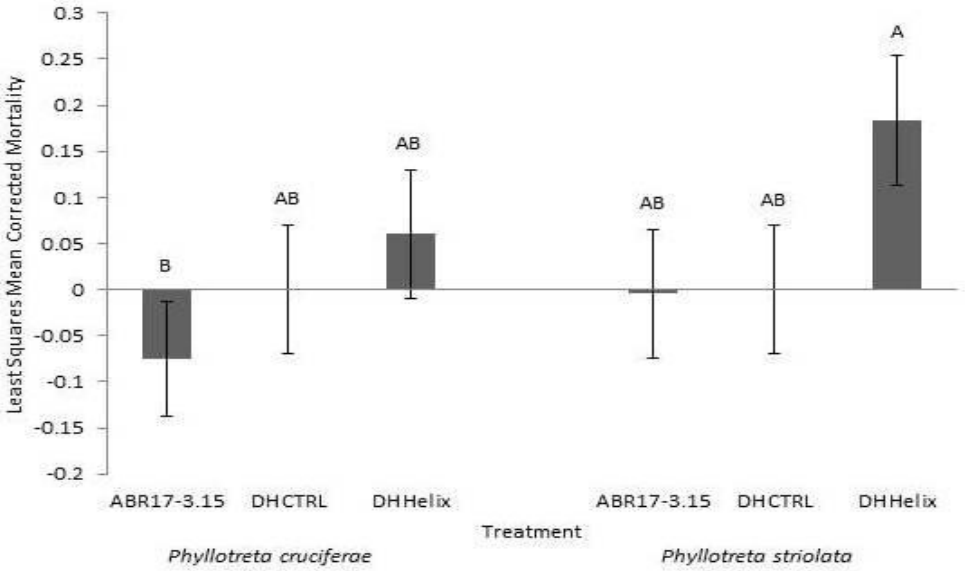

Figure 7. Least squares mean corrected mortality (± S.E.) of the flea beetles *Phyllotreta cruciferae* and *P. striolata* following a 72-hour feeding period on seedlings of *Brassica napus* comprising the doubled haploid control (DHCTRL), the transgenic genotype (ABR17–3.15), and the doubled haploid control seed-treated with thiamethoxam insecticide (DHHelix) at a density of two adults per plant. Letters on histograms indicate significance of differences among treatments: means with the same letter are not significantly different ($P > 0.05$).

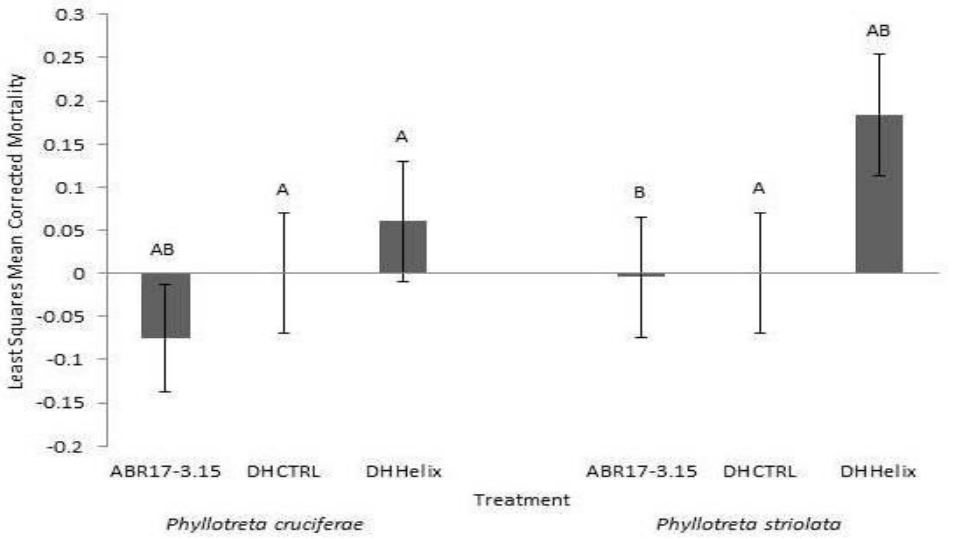

Figure 8. Least squares mean corrected mortality (± S.E.) of the flea beetles *Phyllotreta cruciferae* and *P. striolata* following a 72-hour feeding period on seedlings of *Brassica napus* comprising the doubled haploid control (DHCTRL), the transgenic genotype (ABR17–3.15), and the doubled haploid control seed-treated with thiamethoxam insecticide (DHHelix) at a density of four adults per plant. Letters on histograms indicate significance of differences among treatments: means with the same letter are not significantly different ($P > 0.05$).

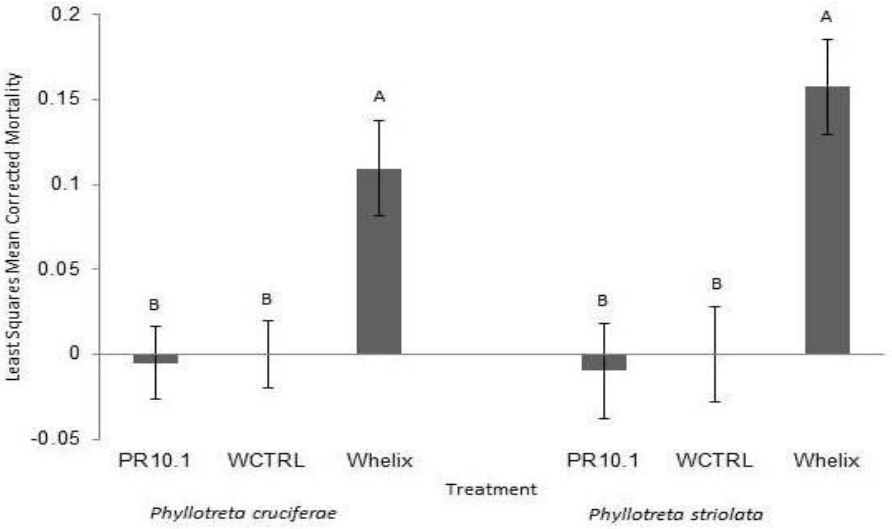

Figure 9. Least squares mean corrected mortality (±S.E.) of the flea beetles *Phyllotreta cruciferae* and *P. striolata* following a 72-hour feeding period on seedlings of *Brassica napus* comprising the Westar control (WCTRL), its transgenic counterpart genotype (PR10.1), and Westar seed-treated with thiamethoxam insecticide (WHelix) at a density of two adults per plant. Letters on histograms indicate significance of differences among treatments: means with the same letter are not significantly different ($P > 0.05$).

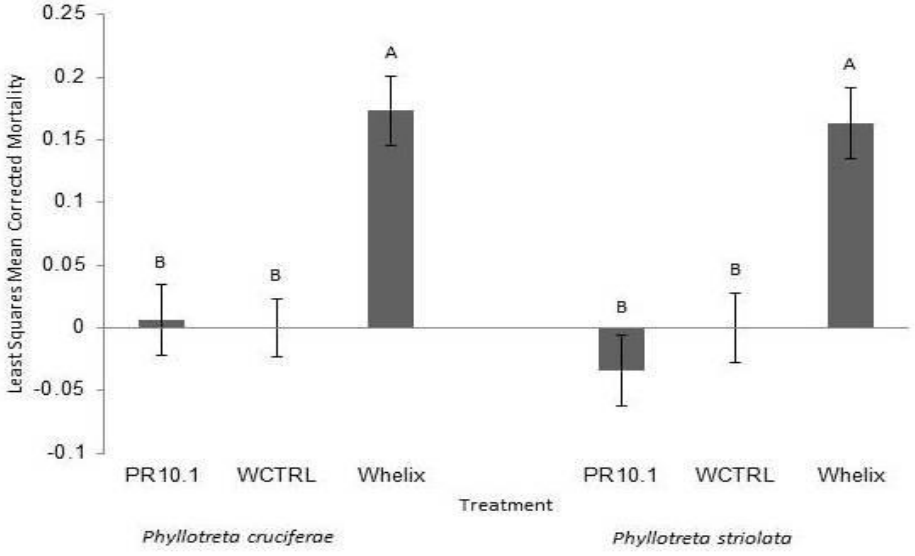

Figure 10. Least squares mean corrected mortality (±S.E.) of the flea beetles *Phyllotreta cruciferae* and *P. striolata* following a 72-hour feeding period on seedlings of *Brassica napus* comprising the Westar control (WCTRL), its transgenic counterpart genotype (PR10.1), and Westar seed-treated with thiamethoxam insecticide (WHelix) at a density of four adults per plant. Letters on histograms indicate significance of differences among treatments: means with the same letter are not significantly different ($P > 0.05$).

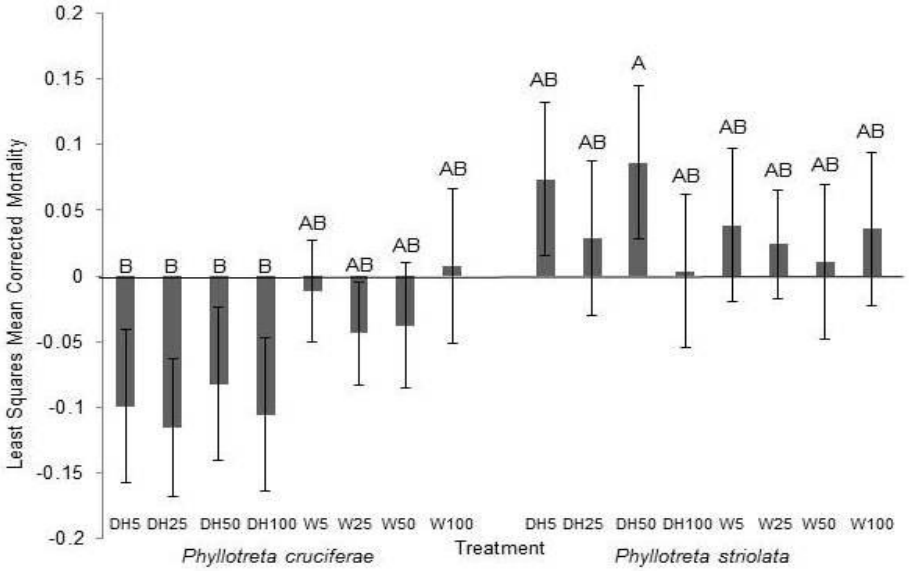

Figure 11. Least squares mean corrected mortality (± S.E.) of the flea beetles *Phyllotreta cruciferae* and *P. striolata* following a 72-hour feeding period on seedlings of *Brassica napus* cv. Westar (W) and doubled haploids (DH) seed-treated with 6-benzylaminopurine at application rates of 5, 25, 50, and 100 µM at a density of two adults of *Phyllotreta* spp. per plant. Letters on histograms indicate significance of differences among treatments: means with the same letter are not significantly different ($P > 0.05$).

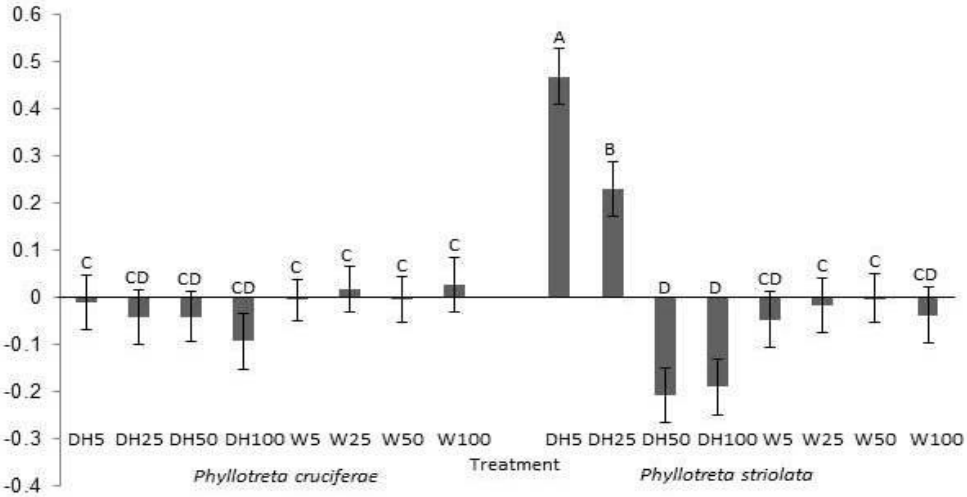

Figure 12. Least squares mean corrected mortality (± S.E.) of the flea beetles *Phyllotreta cruciferae* and *P. striolata* following a 72-hour feeding period on seedlings of *Brassica napus* cv. Westar (W) and doubled haploids (DH) seed-treated with 6-benzylaminopurine at application rates of 5, 25, 50, and 100 µM at a density of four adults of *Phyllotreta* spp. per plant. Letters on histograms indicate significance of differences among treatments: means with the same letter are not significantly different ($P > 0.05$).

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Chapter 3. Herbivory-Induced Proteome Changes in Transgenic *Brassica napus* L. Seedlings Constitutively Expressing Pea ABR17 Upon Feeding by Flea Beetles, *Phyllotreta* spp. (Coleoptera: Chrysomelidae

3.1 Introduction

Canola serves as a valuable Canadian cash crop and generates over $14 billion in economic revenue (Canola Council of Canada 2011). In 2008-2009 China represented Canada's greatest importer of canola seed with 2.87 million tonnes accounting for $1.3 billion (Canola Council of Canada 2011). The crop is grown for its seed, which is rich in oil that is used for human consumption, and protein in the meal that is used for animal feed. Canola is capable of growing at relatively lower temperatures with fewer heat units than other oilseed crops, thus making it an ideal candidate for Canadian growers (Thomas 2002). Canola contains low erucic acid and glucosinolate levels in the seed and is comprised of *Brassica napus* L. and *Brassica rapa* L., which belong to the family Brassicaceae alongside cabbage (*Brassica oleracea* L. var capitata), brown mustard, (*Brassica juncea* (L.) Czern), white mustard (*Sinapis alba* L.), and several other economically important crop species (Downey 1983).

Pathogenesis-related (PR) proteins are proteins that respond to pathogens as well as other stressors and many families of PR proteins have been recognized, including the cytosolic PR10 proteins (van Loon and van Strien 1999). Based on their structure and function, PR proteins are classified under 17 different families (Zubini et al. 2009). PR10 proteins have been shown to be expressed constitutively as well as in response to abiotic and biotic stress conditions. Containing over 100 members, the PR10 protein family was first identified in both mono and dicotyledonous plants in the early 1980s and were originally classified as PR 1 (Maleck et al. 2000; Liu and Xue 2006; Zhijian et al. 2011).

Typically between 155-163 residues in size, these cytoplasmic PR10 proteins have an acidic pH and few similarities among their primary structures. Despite the low sequence identities exhibited by PR10 proteins, they maintain a highly conserved three-dimensional fold comprised of a seven-stranded β-sheet wrapped around a C-terminal α-helix and two additional small α-helices (Markovic-Housley et al. 2003; Zubini et al. 2009). PR10 proteins interact with several plant compounds including cytokinins, brassinosteroids and fatty acids (Fujimoto et al. 1998; Mojensen et al. 2002). Some PR10 proteins have been demonstrated to possess ribonuclease (RNase) activity and this activity may be important for their role in mediating plant stress tolerance (Srivastava et al. 2007). RNA hydrolysis can also be important for the degradation of foreign, pathogenic RNA which could be critical in affording tolerance to biotic stressors (Liu et al. 2003). Recently it has been hypothesized that the RNase activity of PR10 proteins may also lead to an increase in endogenous cytokinin concentrations due to the fact that the constitutive expression of pea (*Pisum sativum* L), ABR17, shown to possess RNase activity, in *Arabidopsis thaliana* leads to an increase in endogenous CK concentrations (Srivastava et al. 2007; Krishnaswamy et al. 2008). It has also been demonstrated that transgenic *A. thaliana* and *B. napus* seedlings expressing pea ABR17 cDNA show enhanced germination and early seedling growth (Srivastava et al. 2007) which may be the result of the observed enhanced endogenous CK concentrations or the result of an as yet uncharacterized activity of PR10 proteins.

Endogenous CKs are also observed to increase during cellular apoptosis and corresponds and correlates with an increase in PR10 protein expression (Zubini et al. 2009). CKs are associated with leaf senescence and ensuring reproductive competence. Located predominantly in areas of high metabolic activity such as plant meristems, CKs are necessary for cell division, regulation of normal growth and development processes as well as possessing

poorly elucidated defensive properties (Matsumoto-Kitano et al. 2008; Nieminen et al. 2008). Natural CKs are N^6-substituted adenine derivatives that typically contain an isoprene-derivative side chain (Haber and Kieber 2002; Hwang and Sakakibara 2005; Rashotte et al. 2003). CK side chains vary in the presence or absence of an hydroxyl group at the terminal and stereo-isomeric position (Hwang and Sakakibara 2005). Synthetic CKs, on the other hand, are derived from diphenylurea and differ structurally from adenine-based CKs (Haberer and Kieber 2002). Further studies are required to elucidate the roles of CKs in mediating plant defense responses; however, it appears that they may possess a role in mediating abiotic stress (Srivastava et al. 2007).

The flea beetles, *Phyllotreta cruciferae* (Goeze) and *Phyllotreta striolata* (Fabricius), are major pests of canola in North America, annually responsible for economic losses due to decreased yields or increased insect control costs. These losses have been estimated at many tens of millions of dollars (Can.) annually (Burgess 1977; Lamb and Turnock 1982; Madder and Stemeroff 1988). Most economic damage occurs when adult beetles invade the crop in spring from their overwintering sites in shelterbelts or wooded areas, and attack newly emerged seedlings by chewing pits in the cotyledons, leaves, and stems (Westdal and Romanow 1972; Burgess 1977). Although some seedlings may recover from flea beetle attack, affected plants can show reduced biomass, delayed maturity, and stunting in their later developmental stages, which affects both seed yield and quality (Putnam 1977; Lamb and Turnock 1982; Lamb 1984). The enhanced seedling vigor demonstrated by plants expressing pea ABR17 cDNA could afford significant benefits to canola seedlings during the over-wintering beetles' spring emergence. The yield of canola may be significantly improved if these seedlings are able to grow to a stage that could adequately tolerate flea beetle damage (Dosdall and Stevenson 2005; Cárcamo et al. 2008).

Because *B. napus* plants constitutively expressing pea ABR17 (PR10.4), specifically the previously characterized ABR17-3.15 line (Dunfield et al. 2007), and those expressing PR10.1 (Srivastava et al. 2004), exhibit enhanced early seedling growth, we wanted to investigate whether these transgenic lines were better at tolerating flea beetle damage. Furthermore, in order to gain an understanding into the molecular mechanisms underlying any observed differences in tolerance to flea beetle damage between these transgenic and non-transgenic lines, we have also characterized herbivory-induced proteome changes in the ABR17-transgenic lines before and after damage. Our results are discussed within the context of the potential utility of PR10 genes in engineering flea beetle tolerance as well as the underlying mechanisms resulting in the observed tolerance.

3.2 Materials and Methods

3.2.1 Seed Treatments and Plant Material

Tested ABR17-3.15 and PR10.1 lines were those previously described by Dunfield et al. (2007) and Srivastava et al. (2004), respectively. Seed treatments included: (1) doubled haploid (DH) and Westar controls which had no insecticide or cytokinin (6-benzylaminopurine) treatment; (2) transgenic DH lines constitutively expressing pea *ABR17* cDNA (ABR17-3.15; Dunfield et al. 2007) and transgenic Westar *B. napus* constitutively expressing pea *PR10.1* cDNA (Srivastava et al. 2004) (3) DH and Westar lines imbibed in 5 µM BAP; (4) DH and Westar lines imbibed in 25 µM BAP; (5) DH and Westar lines imbibed in 50 µM BAP; (6) DH and Westar lines imbibed in 100 µM BAP; (7) DH and Westar lines with Helix® treatments. Concentrations of Helix® were not measured as in previous Tansey et al. (2008; 2009) studies. Helix® applications for our study were not based on concentrations provided by the manufacturer, but rather coating of the seeds with the Helix®.

All seeds underwent surface sterilization and seed splitting procedures to ensure uniformity upon seeding. Surface sterilization of the seeds was conducted to remove the waxy outer layer of the seed coat to increase the uptake of 6-benzylaminopurine (BAP). Seeds of the *B. napus* DH, Westar, PR10.1 or PR10.4 lines were counted in batches of 100 and placed in 1.5 mL tubes. To remove the waxy surface layer, 1 mL of 70% ethanol was applied to the seeds for 20 minutes. After this time the ethanol was removed with a pipette and washed three times, 1 minute each, with autoclaved double distilled H_2O (ddH_2O). To sterilize the seed surface, 20% bleach was applied after the removal of the water, and the seeds incubated for 10 minutes, following which the bleach was removed and the seeds were washed again with autoclaved ddH_2O as indicated earlier. Autoclaved ddH_2O was added to the seeds and they were incubated at room temperature for 24 hours to promote seed splitting. After 24 hours, the autoclaved ddH_2O was removed and replaced with concentrations of BAP of 0, 5, 25, 50 and 100 µM for three hours. Seeds were then washed three times with autoclaved ddH_2O and used for seeding.

Seeds were grown under controlled thermal (21 +/- 2°C) conditions and an 18 hour photoperiod in the greenhouse at the University of Alberta, Edmonton, AB. Seven-dram (25.88 mL) plastic cylindrical vials, with perforated bottoms to allow for water uptake from below, were held together in groups of 10 and potted with commercial potting mix, Sunshine Professional®, Sungro Horticulture. Sunshine Professional® contains fertilizer, in addition to sphagnum peat moss, a wetting agent and lime. The seeds were sown individually in the vials, placed in the greenhouse and watered daily over the course of 7 days.

3.2.2 Trial Setup and Damage Assessment

Trials and assessments were run as per Tansey et al. (2008) and Palaniswamy et al. (1992). Beetle specimen collections were made in canola fields in and around Edmonton, AB,

Canada, including the emerging overwintered generation from May-June 2009 and 2010 and the second generation from August-September 2009 and 2010. After 7 days the most robust seedlings with fully expanded cotyledons were selected. Sixteen seedlings were placed in a 4x4 formation in the centre of a 60 x 60 x 90 mesh cage with no space between the adjacent plants. Beetles were introduced to the caged seedlings within 1 week of capture. Since densities of both *Phyllotreta* spp. of two per seedling and four per seedling are routinely observed in Europe and North America, similar densities were used for this study. There were a total of four trial combinations including *P. cruciferae* at a density of 2 per plant, *P. cruciferae* at a density of 4 per plant, *P. striolata* at a density of 2 per plant and *P. striolata* at a density of 4 per plant. Every trial was conducted with every seed treatment at 4 replicates per treatment. The trials were run for 72h, after which the flea beetles were removed, their mortality recorded and seedlings scored on a defoliation scale of 0-10 similar to the Palaniswamy et al. (1992) method. A measure of 0 corresponded to no defoliation, 1 with 1-10% and 10 with 91-100% defoliation. Experiments were performed in University of Alberta, Edmonton laboratories with ca. ambient light 16:8 light : dark mid-May; ca. 15:9 L : D mid-August (Tansey et al. 2008). Low numbers of *P. striolata* during the 2009 field season resulted in nearly all *P. striolata* assessments being conducted during the spring/summer 2010 field season.

3.2.3 Protein Extraction

Protein was extracted from flea beetle challenged *ABR17-3.15* transgenic plants and controls which was not exposed to the insects in order to characterize the herbivory-induced proteome changes in these lines. Pooled *B. napus* leaves (~300 mg) were ground to a fine powder in liquid nitrogen. Ground leaves were resuspended in 1.5 mL of acetone containing 10% tricholoroacetic acid (TCA; Fisher, Fair Lawn, NJ) and 0.07% ditrhiothreitol (DTT, Fisher). The

suspension was incubated at -20°C for 1 h, vortexed and centrifuged at 18, 000 *g*, for 15 min, at 4°C. The supernatant was removed and the pellet was resuspended in 1 mL ice-cold acetone containing 0.07% DTT and centrifuged as described above. This washing step was repeated four more times. Washed pellets were dried for 10 min in a Speedvac (Hetovac VR-1; Heto Laboratory Equipment A/S, Birkerod, Denmark) and the proteins resolubilized in 400 µL rehydration/sample buffer (Bio-Rad, Mississauga, ON, Canada) containing 0.1% tributylphosphine (TBP, Bio-Rad) and incubated at 4°C overnight. After incubation, the samples were vortexed vigorously and centrifuged as described above. The supernatants were transferred to fresh tubes and stored at -20°C until electrophoresis. Protein samples were prepared twice from two independent undamaged and treatment samples. Protein concentrations were determined using a modified Bradford assay (Bradford 1967) with bovine serum albumin (BSA; Pierce Biotechnology, Rochford, IL) as the standard as described previously.

3.2.4 Two-Dimensional Electrophoresis (2-DE)

Extracted proteins were subjected to first dimension isoelectric focusing (IEF) and second dimension separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (Subramanian et al. 2005; Sharma et al. 2006; Liang et al. 2008). Briefly, 17 cm Immobilized pH Gradient (IPG; pH 4-7, Bio-Rad) strips were rehydrated overnight with 400 μg of protein in 300 µL of rehydration/sample buffer. IEF was performed on a PROTEAN IEF cell (Bio-Rad) to separate proteins based on differences in isoelectric points. The focused IPG strips were equilibrated in 5 mL of equilibration buffer (containing 6 M urea, 2% sodium dodecyl sulfate (SDS), 0.37 M Tris-HCl, pH 8.8, 20% glycerol and 130 mM DTT) twice at 10min each. IPG strips were then incubated in the same buffer containing 135 mM

iodoacetamide (IAA) for 10 min and this tep repeated once more. For the separation of the focused proteins in the second dimension (GEL PERCENTAGE?) , SDS-PAGE was performed using PROTEAN II XI cell (Bio-Rad). Separated proteins were stained using Colloidal Coomassie Blue Staining Kit (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions to visualize the protein spots.

3.2.5 Two-Dimensional Gel Analysis and Protein Identification

Images of the 2-dimensional (2-D) gels were acquired using a GS-800 calibrated densitometer (Bio-Rad) and analyzed with PDQuest 2-D analysis software (version 7.3.1, Bio-Rad) as described in Liang et al. (2008). Matchsets were created from four gels (two biological replicates and two technical replicates for each treatment). Analysis was performed using automated detection and matching tool of the PDQuest software. Artifacts, undetected spots and mis-detected spots were corrected manually. Replicate groups for undamaged and damaged gels were created and analyzed using PDQuest's Student's *t*-test feature in which differential intensities were measured at a significance level of $P < 0.05$. Quantities of statistically significant spots were determined using the spot quantification tool and fold-changes calculated (Liang et al. 2008). Of the statistically significant spots obtained, only those showing reproducible changes (up- or down-regulation) in all replicates were selected to undergo LC/MS/MS analysis at the Institute for Biomolecular Design (University of Alberta, Edmonton, Alberta, Canada).

Stained spots were excised and an automated in-gel tryptic digestion was performed on a Mass Prep Station (Micromass, UK). The gel pieces were de-stained, reduced with DTT, alkylated with iodoacetamide, digested with trypsin (Promega Sequencing Grade Modified) and the resulting peptides extracted from the gel and analyzed via LC/MS/MS. Mass spectrometry

was performed on an Agilent 1100 nano HPLC coupled with a LCQ Deca XP ion trap mass spectrometer (Thermo Scientific, USA). Tryptic peptides were separated using a water/methanol gradient (0.2% formic acid) on a Picofrit reversed-phase capillary column (5 micron BioBasic C18, 300 Angstrom pore size, 75 micron ID x 10 cm, 15 micron tip) (New Objectives, MA, USA), with an in-line trapping column (C18, 300 micron ID x 5 mm) (LC Packings, CA, USA) used as a loading/desalting column. Protein identification from the generated MS/MS data was performed by searching the NCBI non-redundant database using Mascot Daemon (Matrix Science, UK). Search parameters included carbamidomethylation of cysteine, possible oxidation of methionine and one missed cleavage per peptide.

3.2.6 Statistical Analysis

Gels were grouped and compared with protein extraction gels from undamaged leaves using the Student's *t*-test feature in the PDQuest software (Bio-Rad). Statistical analyses of proteome level changes were performed using the Student's *t*-test at a significance level of $P < 0.05$ in the PDQuest software as previously described.

3.3 Results and Discussion

3.3.1 Protein Changes and Identification of Phyllotreta spp. Responsive Proteins

As described previously in this thesis, among the two transgenic lines tested (PR10.1 and ABR17), only the ABR17 seedlings exhibited a significant tolerance to *Phyllotreta* spp. challenge. Therefore, in order to understand the molecular mechanisms which may be mediating the observed tolerance in the ABR17-3.15 transgenic lines, a study of the proteome changes in response to insect challenge was conducted. A total of 1020 protein spots could be detected on gels representing the leaf proteome of *B. napus* (ABR17-3.15) plants (Figure 13). A total of 101

of these spots were initially detected by the automated analysis of gels by PDQuest as exhibiting statistically significant ($P \leq 0.05$) increases or decreases in abundance brought about by *Phyllotreta* spp. challenge. Of these 101 proteins, changes in abundance of 63 proteins were confirmed to be reproducible through manual verification and were selected for MS/MS analysis which resulted in 33 identifications (Table 1). Most protein spots were identified as a single protein; however, a few came back with multiple "hits". In those cases, molecular weights (molecular masses and isoelectric points), Mascot scores, number of peptides and percentage of amino acid coverage in the peptides were used to try and establish the identity of the protein (Liang et al. 2008).

3.3.2 Proteins of interest

As stated earlier, a number of proteins were observed to increase in abundance in response to the insect treatment in *ABR17-3.15 B. napus* transgenic plants. Many proteins involved in metabolic pathways were identified (Table 1) which may be contributing to the observed enhanced seedling growth as previously described (Srivastava et al. 2007). We have selected a few proteins from the ones reported in Table 1 as perhaps having a role in mediating the observed tolerance to insects.

The first one was spot #3 which was identified as a cysteine protease from *B. rapa* var. perviridis, exhibiting a significant increase in fold change of 2.10 ± 0.86 in samples that underwent herbivory damage over the undamaged samples. Another protein related to its role in proteolysis identified in our study belonged to the group of trypsin inhibitors (14 and 15) which exhibited a significant increase in abundance. Spots #14 and 15 exhibited fold changes unique to ABR17-3.15 that underwent flea beetle herbivory (Table 1). The second group of proteins exhibiting significant increase in abundance were identified as *Brassica napus* drought

22 kD (BnD22) induced protein (spots #12 and 13) which exhibited an extreme increase in abundance as a result of insect challenge of 2017.96 ± 815.05 and 564.88 ± 346.36 respectively (Table 1). The possible roles of these proteins in mediating the observed insect tolerance are discussed in subsequent paragraphs.

Defoliated doubled haploid transgenic, ABR17-3.15, displayed significantly lower feeding damage than defoliated DH control. ABR17-3.15 exhibited no statistically significant differences in feeding damage when compared with Helix®-treated DH. Since the ABR17-3.15 transgenic performs at the same level as the Helix®-treated DH seeds, as previously reported in Chapter 2 section 3.1.1, there must be some physiological differences underlying the observed antixenosis properties. Experiments conducted to determine herbivory-induced molecular changes by *P. cruciferae* and *P. striolata* determined that a total of 101 protein spots changed significantly in abundance when compared with seedlings not subjected to herbivory. MS/MS analysis of 63 out of the 101 spots resulted in the identification of 33 proteins. Sharma et al. (2006) and Srivastava et al. (2007) demonstrated a number of metabolic enzymes exhibiting increased expression in *B. napus* when exposed to the fungal pathogen *Alternaria brassicae* and abiotic stress conditions, respectively. This study is the first to demonstrate similar metabolic changes in *B. napus* as a result of insect herbivory. These proteins could be associated with enhanced seedling vigor and overall growth and development. More interesting, however, was the increased abundance of proteases and trypsin inihibitor proteins and drought-induced protein (Table 1). Their possible roles in mediating the observed tolerance are discussed below.

Proteases have been identified as having possible cytotoxic capabilities (Pechan et al. 2002; Mohan et al. 2006) while trypsin inhibitor proteins have been shown to be involved in biotic (Hilder et al. 1987) and abiotic resistance (Srinivasan et al. 2009). Cysteine proteases are associated with a number of cellular functions, including autolysis during programmed cell

death, and have been known to play a role in herbivory defense (Pechan et al. 2002; Mohan et al. 2006). Spot #3 from Table 1 demonstrated increased expression of cysteine protease. Spot #3 was identified as cysteine protease with significantly increased abundance after being exposed to *Phyllotreta* spp. feeding damage. It displayed an experimental and theoretical M_r/p*I* of approximately 27000/4.4 and 48570/4.82 respectively (Table 1). The differences between experimental and theoretical M_r/p*I* values may be the result of protein degradation during processing of samples.

Increased abundance of cysteine proteases may confer antixenosis properties; however, with little research conducted on this hypothesis, it is difficult to conclude as much. It is clear from the literature that cysteine proteases may have a role in insect tolerance; for example, maize insect resistance 1-cysteine protease (Mir1-CP) from insect-resistant maize was previously identified as a plant defense mechanism against insect herbivory (Pechan et al. 2002; Lopez et al. 2007). Mir1- CP was shown to disrupt the peritrophic membrane within the insect midgut, thereby interfering with its growth and development (Pechan et al. 2002; Lopez et al. 2007). Since an increase in cysteine protease abundance was shown in damaged plants, it is suggestive that it may play an antixenosis role in ABR17-3.15. A preference feeding trial would help validate this hypothesis. Although the mechanism for our cysteine protein has not been elucidated, the precedence of cysteine proteases functioning as plant defense molecules perhaps adds a strong indication to the validity of our argument.

Protease inhibitors are known to act as defensive compounds against biotic (Hilder et al. 1987; Chen et al. 1999) and abiotic stresses (Srinivasan et al. 2009). Although this current study identified two trypsin inhibitors through mass spectrometry analysis (spots #14 and 15), previous studies have highlighted our identified BnD22 proteins (spots #12 and 13) as performing a dual functionality as a water-soluble chlorophyll-binding protein (Nishio and Satoh,

1997; Satoh et al. 2001) and a trypsin inhibitor (Desclos et al. 2008). The first BnD22 drought-induced protein, spot #12, displayed experimental and theoretical M_r/p*I* of approximately 14900/5.0 and 23690/5.89 respectively. The second BnD22 protein, spot #13, resulted in experimental and theoretical M_r/p*I* of approximately 14600/5.0 and 23690/5.89 respectively. Spot #14, the trypsin inhibitor peptide, demonstrated experimental and theoretical M_r/p*I* of approximately 18400/5.1 and 23534/5.1 respectively. Meanwhile, the second trypsin inhibitor peptide, spot #15, displayed experimental and theoretical M_r/p*I* of approximately 18600/5.1 and 23345/5.1 respectively (Table 1). The experimental and theoretical M_r/p*I* for spots #14 and 15 appear to validate the identity of the proteins, while the reduced M_r/p*I* of the BnD22 proteins may be the result of protein degradation through sample processing. The large fold change observed for spots #12, 13 indicates that these spots were expressed almost exclusively in ABR17-3.15 which experienced flea beetle herbivory damage. Similarly the uniqueness of the expression of spots #14 and 15 indicate that that they are somehow involved in conferring tolerance against insect herbivory.

The double function of BnD22 proteins is believed to provide protection for younger tissues under stress conditions by maintaining protein integrity and photosynthesis capabilities (Desclos et al. 2008). This may explain ABR17s ability to perform well under abiotic stress conditions and may also somehow be involved in conferring antixenosis capabilities. To the best of my knowledge there have been no previous studies involving BnD22 and its association with conferring insect resistance. Previous studies involving transgenic tobacco expressing a cowpea trypsin inhibitor gene showed an increase in insect resistance (Hilder et al. 1987). Similarly, overexpression of corn trypsin inhibitor in *Escherichia coli* (Migula 1895) Castellani and Chalmers inhibited the growth of pathogenic plant fungi (Chen et al. 1999). In conjunction with the increased expression of cysteine proteases, the increased expression of trypsin inhibitors may

potentially compound the antixenosis effect of the ABR17-3.15 transgenic. Since trypsin inhibitors have also been shown to have increased expression under drought and salt stress (Kim et al. 2001), their role in biotic plant defense cannot be discounted.

This study investigated a transgenic *B. napus* genotype that exhibited reduced feeding damage but this warrants further research in order to elucidate and the mechanisms involved in conferring its antixenosis properties. Similarly, a greater number of technical replicates could have been performed to increase the precision of the molecular weights and misreadings from the PDQuest software. Nevertheless, results presented here have enormous potential on future pesticide applications in agroecosystems. Future studies should focus on BnD22 expression and the potential for conferring insect resistance. With proper insect resistance management protocols and implementation, the longevity of the novel proteins and genes present in ABR17-3.15 can be maintained and contribute toward insect resistance. The observed increase in antixenosis in the transgenic genotype ABR17-3.15 could result in a drastic reduction in seed-applied and field-applied insecticides. Also, with the enhanced seedling vigor previously recorded from ABR17-3.15, the genotype has the potential of performing significantly better than other varieties under more severe abiotic stresses such as salinty and drought. Further studies are needed to verify the specific mode of action of the antixenotic properties associated with this new transgenic genotype.

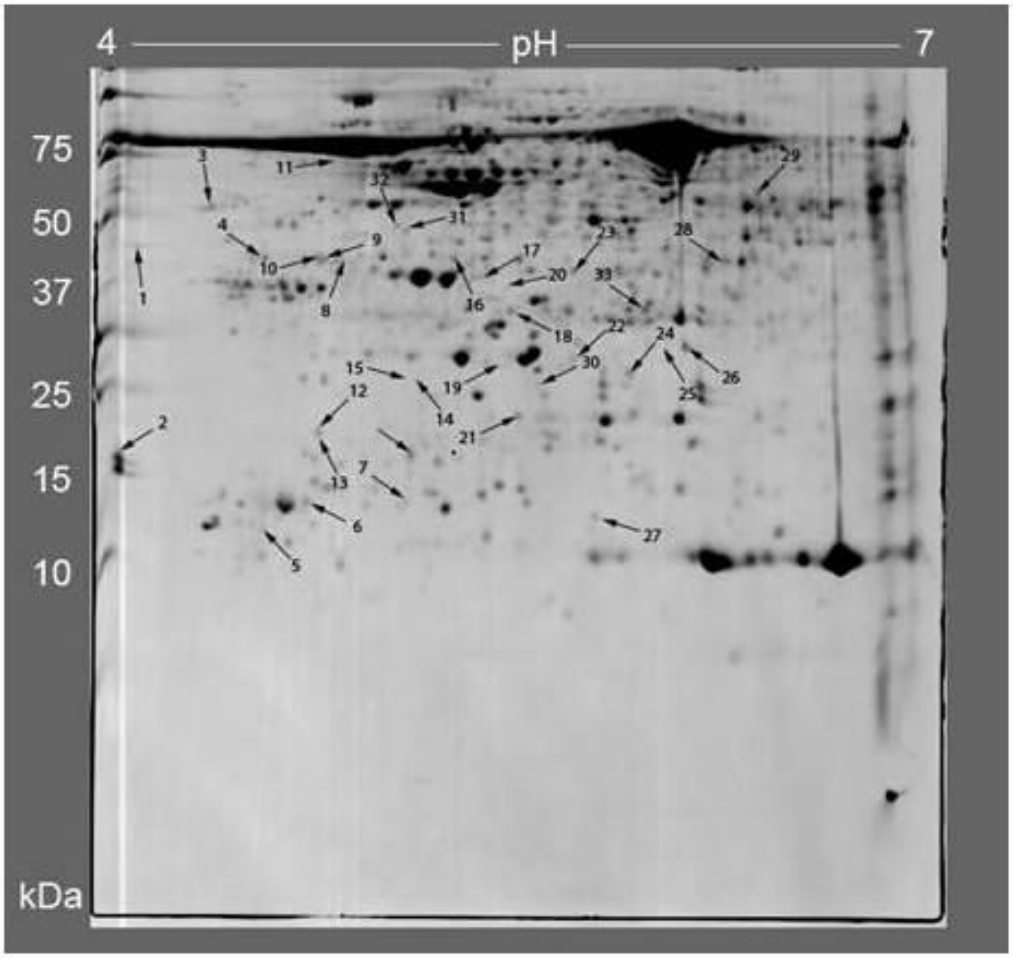

Figure 13. Representative image of proteome level analysis of undamaged *B. napus* leaf proteins separated by 2-DE and visualized using Coomassie Blue (arrows indicate protein spots selected for MS/MS analysis at $P < 0.05$ as a result of flea beetle damage).

Table 1: Identities of proteins from *ABR17-3.15 B.napus* whose abundance increased significantly as a result of *Phyllotreta* spp. challenge.

spot	protein identity	significance		MS/MS /PM[a] (%)	score[b]	sequence	accession no.[c]	M_r/p*I*	fold change[d]
		UD	DAM						
1	Vacuolar calcium binding protein (*Raphanus sativus*)		*	3/12	97	K.EEEAPVEVTTK.D K.TEEVVEPK.K K.TEETPAVVEEEK.K	gi|9049359	27094/4.12	+4.21 ± 4.38
2	Eukaryotic translation initiation factor-5A (*Brassica napus*)		*	3/21	79	K.TYPQQAGNIR.K K.CHFVAIDIFTAK.K K.LPTDDNLSALMK.S	gi|40805177	17315/5.71	+3.44 ± 1.46
3	Cysteine protease (*Brassica rapa* var. perviridis)		*	4/14	192	K.NGGIDTEEDYPYK.G K.VVTIDSYEDVPANSEESLKK.A K.ALSHQPISVAIEGGGR.A K.CGIAVEPSYPIK.N	gi|171702843	48570/4.82	+2.10 ± 0.86
4	eEF-1beta (*Arabidopsis thaliana*)		*	1/5	73	R.SIQMEGLFWGASK.L	gi|398606	25193/4.53	+4.42 ± 0.15
5	Ribulose bisphosphate carboxylase large chain (*Brassica oleracea*)		*	4/9	215	R.ESTLGFVDLLR.D R.DLAVEGNEIIR.E K.WSPELAAACEVWK.E K.EITFNFPTIDK.L	gi|1346967	53436/5.88	+3.42 ± 0.22
6	Ribulose bisphosphate carboxylase large chain (*Brassica oleracea*)		*	6/13	322	K.LNYYTPEYETK.D K.DTDILAAFR.V R.ESTLGFVDLLR.D R.DLAVEGNEIIR.E K.WSPELAAACEVWK.E K.EITFNFPTIDK.L	gi|1346967	53436/5.88	+2.98 ± 0.19
7	ABA-responsive protein ABR17 (*Pisum sativum*)		*	3/33	130	M.GVFVFDDEYVSTVAPPK.L K.EAQGVEIIEGNGGPGTIK.K K.VAFETIILAGSDGGSIVK.I	gi|1703042	16619/5.07	+2.85 ± 0.50
8	PSBO1 (PS II OXYGEN-EVOLVING COMPLEX 1) (*Arabidopsis thaliana*)		*	3/13	155	K.GTGTANQCPTIDGGSETFSFK PGK.Y R.VPFLFTVK.Q K.NTAASVGEITLK.V	gi|15240013	35349/5.55	+5.49 ± 4.92

9	Uridylyltransferase-related (*Arabidopsis thaliana*)		*	4/15	222	R.LGALLDTMNALK.N K.NLGLNVVK.A K.VEDPELLEAIR.L K.ALIKPLQQVLANSLR.Y	gi|18394414	31446/4.98	+5.40 ± 3.14
10	Fibrillin (*Brassica napus*)		*	2/11	135	R.AEISELITQLESK.N K.GLLTSVQDTASSVAR.T	gi|4139097	25858/5.12	+2.17 ± 0.43
11	Hypothetical protein ARALYDRAFT_482998 (*Arabidopsis lyrata*)		*	7/21	253	K.GLAYDTSDDQQDITR.G K.SFQCELVMAK.M K.MGINPIMMSAGELESGNAGEPAK.L K.MCCLFINDLDAGAGR.M K.FVEGLGVEK.I R.EGPPVFEQPEMTLEK.L K.LMEYGNMLVMEQENVK.R	gi|297827581	52208/5.69	+3.87 ± 0.34
12	BnD22 drought induced protein (*Brassica napus*)		*	2/13	107	K.LWAVDVSSSAAK.E K.EPAIIIGGESTAPNSLFK.I	gi|17813	23690/5.89	+2017.96 ± 815.05
13	BnD22 drought induced protein (*Brassica napus*)		*	3/18	150	K.LWAVDVSSSAAK.E K.EPAIIIGGESTAPNSLFK.I K.IEEATEANTYK.L	gi|17813	23690/5.89	+564.88 ± 346.36
14	Trypsin inhibitor propeptide (*Brassica oleracea*)		*	3/20	139	R.GNQCPLFIGQER.S R.VGFVPEEENLNIK.M R.SLFIAAGPKPEAGGEDSSR.S	gi|841208	23534/5.12	Unique
15	Trypsin inhibitor propeptide (*Brassica oleracea*)		*	3/20	110	R.GNQCPLFIGQER.S R.VGFVPEEENLNIK.M R.SLFIAAGPKPEAGGEDSSR.S	gi|841208	23534/5.12	Unique
16	Fructose-1,6-bisphos-phatase (*Arabidopsis lyrata*)		*	1/3	64	K.KLDVLSNDVFVK.A	gi|1169585	37589/5.31	+4.05 ± 1.76
17	PSBO1 (*Arabidopsis thaliana*)		*	5/16	150	K.GTGTANQCPTIDGGSETFSFKPGK.Y K.KFCFEPTSFTVK.A	gi|15240013	35349/5.55	+2.17 ± 0.73

						K.FCFEPTSFTVK.A R.VPFLFTVK.Q K.NTAASVGEITLK.V			
18	Triosephosphate isomerase (*Secale cereal*)		*	2/8	94	K.VAYALAQGLK.V K.VIACVGETLEQR.E	gi|1174749	27138/5.24	+2.01 ± 0.38
19	AT5g66570/K1F13_25 (*Arabidopsis thaliana*)		*	4/13	138	K.GTGTANQCPTIDGGSETFSFK PGK.Y K.KFCFEPTSFTVK.A K.FCFEPTSFTVK.A R.VPFLFTVK.Q	gi|13926291	35335/5.55	+6.91 ± 2.07
20	Dinitrogenase reductase (uncultured *Frankia* spp.)		*	1/5	57	R.DDELIMELAR.R	gi|212293181	19678/4.73	+3.54 ± 0.97
21	Hypothetical protein (*Vitis vinifera*)		*	4/22	168	K.ELENVPMVTTESGLQYK.D K.GLDEGILTMK.T R.RIYIPGPLAFPK.G R.IYIPGPLAFPK.G	gi|225434038	18656/5.03	+2.42 ± 0.57
22	Photosystem II reaction center PsbP family protein (*Arabidopsis lyrata*)		*	4/16	216	R.EYIDTFDGYSFK.Y R.QYLTEFMSTR.L K.SYANNNELAVMPQDR.V R.YLAVLGVENNR.L	gi|297804700	32299/8.95	+2.86 ± 1.24
23	Putative protein (*Arabidopsis thaliana*)		*	3/8	135	R.KAEQYLADSGTPYTIIR.A K.AEQYLADSGTPYTIIR.A R.ELLVGKDDELLQTDTK.T	gi|7340698	41856/6.05	+2.15 ± 0.35
24	Fe-superoxide dismutase (*Arabidopsis thaliana*)		*	1/4	66	K.PSGELLALLER.D	gi|166700	25409/6.30	+18.14 ± 3.83
25	Superoxide dismutase (*Raphanus sativus*)		*	4/19	124	K.QTLEFHWGK.H K.PSGELLALLER.D R.DFTSYEK.F K.TFMNNLVSWEAVSSR.L	gi|3114705	23791/5.96	+22.49 ± 6.22

26	Superoxide dismutase (*Raphanus sativus*)		*	6/28	246	K.QTLEFHWGK.H R.AYVDNLKK.Q K.QVLGSELEGK.A K.PSGELLALLER.D R.DFTSYEK.F K.TFMNNLVSWEAVSSR.L	gi|3114705	23791/5.96	+9.51 ± 5.5
27	ATP synthase CF1 epsilon subunit (*Arabidopsis thaliana*)		*	6/39	203	M.TLNLCVLTPNR.I R.IVWDSEVK.E R.IGNNEITILVNDAEK.N K.RQTIEANLALR.R R.QTIEANLALR.R R.VEALNTI.-	gi|7525039	14547/5.84	+4.21 ± 1.15
28	Glyoxylase II (*Arabidopsis thaliana*)		*	1/4	64	K.VSIGDIYLEVR.A	gi|1644427	27941/5.58	+7.78 ± 4.75
29	Predicted protein *(Physcomitrella patens* subsp. patens)		*	1/4	73	R.ALQESIASELAAR.M	gi|168012869	33432/5.75	+4.87 ± 1.17
30	Putative delta subunit of ATP synthase (*Brassica rapa*)		*	5/37	244	R.LVIDEIVK.S K.ITDTQLAEVR.S K.LEPPQLAQIAK.Q K.TVLDPSLVAGFTIR.Y K.LIDMSVK.K	gi|1480014	14862/9.19	+12.32 ± 3.26
31	Hypothetical protein ARALYDRAFT_329544 (*Arabidopsis lyrata* subsp. lyrata)		*	2/10	128	K.VVETYEATSAEVK.A K.VCVFLPEEVK.T	gi|297804120	24725/4.91	+3.05 ± 0.73
32	Hypothetical protein ARALYDRAFT_329544 (*Arabidopsis lyrata* subsp.		*	2/10	123	K.VVETYEATSAEVK.A K.VCVFLPEEVK.T	gi|297804120	24725/4.91	+2.86 ± 0.94

	lyrata)								
33	Malate dehydrogenase (*Micromonas* sp. RCC299)	*		4/12	132	K.GCDLVIIPAGVPR.K K.KIFGVTTLDVVR.S K.IFGVTTLDVVR.S K.AGAGSATLSMAYAAAR.M	gi|255073915	33823/4.95	-2.34 ± 0.46

UD: Undamaged seedlings; **DAM**: Damaged seedlings

***** - Indicates statistical significance at $P < 0.05$

[a] Number of peptides matched/percent sequence coverage.

[b] Mascot score for the most significant hits.

[c] Accession numbers for proteins generated by the Mascot search.

[d] Fold change = (Average normal quantity of damaged seedling) / (Average normal quantity of undamaged seedling)

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Chapter 4. General Discussion

Pathogenesis related (PR) 10 proteins belong to the 10th member of a total 17-member group of identified PR proteins (van Loon et al. 1994; van Loon and van Strien 1999). PR10 proteins increase in abundance during biotic and abiotic stress conditions including those caused by fungal and viral pathogens, drought and heavy metal stress (Liu and Ekramoddoullah 2006). Enhanced PR10 protein expression has also produced increased tolerance and early seedling vigor enabling plants to better withstand abiotic stress, such as salt stress and cold temperatures (Kav et al. 2004; Srivastava et al. 2004; Liu et al. 2005; Srivastava et al. 2006; Srivastava et al. 2007; Chevalier et al. 2008; Zubini et al. 2009). They have been shown to interact with a number of important phytohormones including cytokinins, brassinosteroids and fatty acids (Srivastava et al. 2007; Chevalier et al. 2008; Fernandes et al. 2008). It is evident from the body of research available in the literature that PR10 proteins possibly play multiple roles *in planta*, some of which are, as of yet, uncharacterized.

Cytokinins are located predominantly in areas of high metabolic activity such as in plant meristems. Cytokinins are necessary for cell division, and regulation of normal growth and developmental processes. In addition, cytokinins confer defensive properties although these remain poorly elucidated (Matsumoto-Kitano et al. 2008; Nieminen et al. 2008). Cytokinins have been shown to increase in abundance in response to PR10 expression (Srivastava et al. 2007).

Studies in the Kav laboratory at the University of Alberta, Edmonton, AB have demonstrated that transgenic *Arabidopsis thaliana* (L.) Heynh. and *Brassica napus* L. seedlings constitutively expressing pea (*Pisum sativum* L.) ABR17 (PR10.4) cDNA showed enhanced germination, early seedling growth and earlier flowering in *B. napus* which may result in elevated endogenous CK concentrations as a result of its observed RNase activity or as of yet uncharacterized activity (Dunfield et al. 2007; Srivastava et al. 2007; Krishnaswamy et al. 2008).

In fact, ABR17, shown to possess RNase activity, and as indicated earlier the expression of *ABR17* cDNA in *A. thaliana* leads to an increase in endogenous CK concentrations which may be the direct result of the degradation of CK containing tRNA molecules (Srivastava et al. 2007; Krishnaswamy et al. 2008). Similarly, an isoform of the PR10 protein family, PR10.1, expressed in *B. napus* (cv. Westar) was found to enhance germination and early seedling vigor under normal and saline conditions (Kav et al. 2004; Srivastava et al. 2004; Srivastava et al. 2006). It was these properties of some members of the PR10 protein family that prompted our studies to investigate whether the early seedling growth and vigor induced in canola seedlings could enhance their tolerance to attack by the principal insect pests of canola seedlings in North America, the flea beetles *Phyllotreta striolata* (Fabricius) and *Phyllotreta cruciferae* (Goeze) (Coleoptera: Chrysomelidae). These insects overwinter as adults in shelterbelt or other protected areas, and emerge in spring with their energy reserves depleted (Westdal and Romanow 1972; Ulmer and Dosdall 2006; Dosdall and Mason 2010). To build up the nutrient reserves needed for reproduction, they feed voraciously on canola seedlings, causing considerable crop damage and consequent losses in yield and crop quality (Lamb 1984).

The purpose of this study was to: (1) test the ability of *ABR17 (PR10.4)* and *PR10.1*-transgenic canola to withstand attack by both flea beetle species; (2) compare tolerance achieved with the transgenic canola germplasm with that achieved with insecticidal seed treatments; (3) determine the effect of cytokinin treatment on the ability of canola seedlings to tolerate and compensate for flea beetle (*Phyllotreta* spp.) (Coleoptera: Chrysomelidae) attack; and (4) investigate herbivory-induced proteome changes in the transgenic lines before and after feeding damage. My hypothesis was that the novel germplasm will demonstrate greater tolerance to feeding by adult flea beetles, since these transgenic lines produce greater quantities of cytokinins, and consequently show more rapid seedling growth.

An additional research objective was to investigate cytokinin-treated canola seed for enhancing seedling growth, thereby fostering improved tolerance of seedlings to flea beetle attack. Since increased cytokinin concentrations have been linked to increased seedling vigor (Srivastava et al. 2007) and PR protein expression (Zubini et al. 2009), I hypothesized that cytokinin imbibition will confer increased tolerance to flea beetle feeding. Mutants expressing pea ABR17 have yet to be tested against insect pests and the results of this study are the first of their kind.

This study demonstrated that both ABR17-3.15, derived from doubled haploid *B. napus*, and PR10.1, derived from *B. napus* cv. Westar, exhibited significantly less feeding damage from *P. cruciferae* attack at densities of two and four beetles per plant when compared with their wild type counterparts. ABR17-3.15 and PR10.1 confer resistance to *P. cruciferae* by nonpreference or antixenosis according to Painter (1951) and Kogan and Ortman (1978), since increased mortality was not observed in either ABR17-3.15 or PR10.1 feeding trials (Figs. 7, 8, 9, 10). Although treatments of seed with the systemic, neonicotinoid insecticide thiamethoxam (Helix®) caused significantly greater flea beetle mortality than ABR17-3.15 (Figures 7, 8) and PR10.1 (Figures 9, 10) at densities of both two and four beetles per plant and for both *P. cruciferae* and *P. striolata*, the feeding damage provided noteworthy results. Most importantly, ABR17-3.15 (Figure 1) and PR10.1 (Figure 3) exhibited no statistically significant differences in feeding damage when compared with their insecticide-treated wild type counterparts. The results are quite relevant since *P. cruciferae* remains the dominant flea beetle species attacking canola in the Mixed Grassland, Moist Mixed Grassland, and Aspen Parkland Ecoregions of western North America (Dosdall and Mason 2010).

Thiamethoxam is a second generation neonicotinoid-based systemic insecticide currently comprising the most common form of pesticide-based control for *Phyllotreta* spp. in

North America (Elliott et al. 2004; Dosdall 2009). Although thiamethoxam can be quite effective for achieving control of flea beetle infestations in canola (Elliott et al. 2004), significant environmental problems have been associated with its use. Powder dispersion during sowing can affect pollinating insects, such as honeybees, that can be exposed as powder settles on local soil and vegetation (Greatti et al. 2003; Iwasa et al. 2004; Greatti et al. 2006; Tremolada et al. 2010). Further, the annual treatment of many millions of hectares of canola cropland with thiamethoxam is placing considerable selection pressure on flea beetle populations to develop resistance to this compound (L. Dosdall, personal communication). The ability of ABR17-3.15 and PR10.1 to perform equivalently to Helix®-treated seeds for control of *P. cruciferae* adds a positive attribute to the potential use of the transgenic canola seedlings because mortality to non-target and beneficial arthropod species is not associated with the novel genotypes. The results validate my hypothesis and demonstrate the considerable potential of utilizing ABR17-3.15 and PR10.1 in place of insecticide-treated seeds within the context of an integrated management strategy. However, a variety of mechanisms may be involved in the observed tolerance and further studies are needed to elucidate their pattern(s) and process(es).

Canola is grown widely throughout the world in continents that include Europe, North America, South America, Australia, and Asia. Canada is the world's second largest canola producer as of 2009, trailing closely behind China (Food and Agricultural Organization of the United Nations 2009). With the observed cold-tolerance and other observed stress tolerances in *B. napus* expressing the ARB17 gene, the potential exists to expand canola production to new regions (Srivastava et al. 2006). The antixenotic responses of *P. cruciferae* to the transgenic canola (and possibly other insect herbivores) imparts additional potential for expansion of the crop.

As expected, densities of four beetles per plant yielded greater damage than two beetles per plant due to the sheer increase in numbers. With less available plant material per insect, the observed damage was sometimes very extensive. Although outbreak densities of four beetles per plant are uncommon in North American canola production, they can occur if integrated management techniques are not implemented (Hiiesaar et al. 2003; Tansey et al. 2008). A number of methods can be employed to manage flea beetle populations in an integrated management strategy so that crop damage from outbreak densities can be ameliorated. In conjunction with utilizing a resistant canola variety, such as one derived from ABR17-3.15 or PR10.1, fall seeding has been shown to be associated with less feeding damage than spring-sown canola (Dosdall and Stevenson 2005). In addition, widening row spacing and increasing seeding rate have been shown to reduce flea beetle damage (Dosdall et al. 1999; Dosdall and Stevenson 2005). Damage was significantly reduced simply by increasing the seeding rate from 7.5 to 10.0 kg/ha or 12.5kg/ha. An added benefit to managing flea beetle populations with increased seeding rate is that this practice also has the ability to manage root maggot damage and losses due to weeds (O'Donovan 1994; Dosdall et al. 1996; Dosdall and Stevenson 2005). Implementing a zero or reduced tillage regime is a key component in the integrated management of flea beetles because these insects prefer warm, dry environmental conditions. The cool, moist microhabitats developed under reduced tillage favor development of the plant more than activity of the beetles (Dosdall et al. 1999). Other benefits of reduced tillage for flea beetle control, in addition to reducing flea beetle feeding damage, include reducing soil erosion and increasing soil organic matter (Stinner and House 1990; Jensen and Timmermans 1991). Planting seed with a greater seed size and weight, and with germination above 95% favors development of competitive plant stands that can reduce flea beetle feeding damage (Elliott et al. 2007). Intercropping canola with non-host plants like wheat can minimize

flea beetle attack (Hummel et al. 2009), and mixing insecticide-coated seed with untreated seed in a 1:3 ratio can reduce insecticide inputs to the environment while reducing flea beetle damage (Soroka et al. 2008).

Interesting trends were observed for the cytokinin treatment experiments. At two *P. cruciferae* per plant, damage was significantly lower for doubled haploids treated with 25 than 5 µM of 6-benzylaminopurine (BAP), and damage increased between the 50 and 100 µM concentrations, indicating a potential optimum threshold value for conferring resistance (Figure 5). *Brassica napus* cv. Westar displayed a numerical increase in feeding damage with increasing BAP concentrations with no significant reduction in damage observed between 5 and 25 µM of BAP. A somewhat inverted but nonsignificant trend was observed with *B. napus* doubled haploids at a density of two *P. striolata* per plant as damage declined from 5 to 50 µM of BAP, but damage then increased for doubled haploids with 100 µM of BAP (Figure 5). Westar imbibed with cytokinins exhibited similar responses for *P. striolata* as damage significantly declined between 5 to 25 µM of BAP then increased at 50 and 100 µm BAP (Figure 5). Feeding damage to seedlings developing from seed that imbibed BAP at four *Phyllotreta* spp. per plant exhibited no discernable trends as the increased density resulted in extensive feeding damage that obscured treatment effects (Figure 6). Consequently, my hypothesis that cytokinin imbibition will confer increased resistance to flea beetle feeding has not been proven or disproven. As stated, the results seem to indicate that a threshold value of BAP concentration exists with the potential for increasing flea beetle resistance. However, further studies need to be conducted in order to determine this value and whether or not it exists.

It is important to note that although cytokinin treatments may not have yielded definitive results, cytokinins are important phytohormones that regulate plant growth and development. Their ability to promote early seedling vigor and enhance germination alone is an

important consideration when developing an integrated flea beetle management strategy (Srivastava et al. 2007). Fall seeding has been shown to decrease flea beetle damage, and this was attributed to the ability of the seedlings to develop beyond the vulnerable cotyledon stage before attack by flea beetles (Dosdall and Stevenson 2005). However, fall seeding of canola has not been implemented on a large scale because seedlings may germinate too early when warmer than anticipated fall or winter weather causes seed to imbibe water, and after subsequent germination, emerged seedlings are killed due to frost, resulting in a low-density plant stand (Clayton et al. 2004; Dosdall and Stevenson 2005). The adoption of utilizing transgenic canola plants (such as ABR17or PR10.1) with enhanced seedling vigor and germination could enable canola growers to seed in early spring, yet produce a plant stand that can withstand flea beetle damage because seedlings that develop rapidly could outgrow spring-emerging flea beetles. Future field-based trials are required to determine how ABR17 and PR10.1 perform under field conditions. Similarly, quantifying the uptake of cytokinin through mass spectrometry may be useful to validate the phytohormone's uptake. It is assumed that cytokinin uptake is uniform for all seeds; however, ensuring uptake concentrations could be useful in further extrapolating the relationships between cytokinins, feeding damage and flea beetle mortality.

Insecticidal seed treatments caused greater adult beetle mortality than the transgenic seedlings or the controls. Under both flea beetle densities and species, seed treatment with Helix® induced the highest recorded mortality (Figures 7, 8, 9, 10). No significant differences in mortality were observed between beetle species, a result that contradicts previous studies (Tansey et al. 2008; Tansey et al. 2009). This study used Helix® applications performed in the laboratory in petri dishes on seed that had been surface sterilized. Tansey et al. (2008, 2009) utilized seeds that were treated using commercial application technology. This could account for

discrepancies between the quantities of insecticide adsorbed onto the seed coats. However, it is safe to assume that results are reproducible since the Helix®-treated Westar performed similarly to the results observed by Tansey et al. (2008, 2009) and the PR10.1 transgenic performed comparably against it. ABR17 and PR10.1 do not seem to possess insecticidal properties as their mechanism for reduced feeding appears to be antixenotic resistance (Figures 7, 8, 9, 10). Similarly, cytokinin treatments did not demonstrate an increase in flea beetle mortality with increased concentrations. Although increased numerical results were observed for mortality for two *P. striolata* per plant when compared to two *P. cruciferae* per plant, no statistically significant results were recorded (Figure 11). To my knowledge, no previous studies have been conducted testing cytokinins and their influence on herbivorous insect feeding damage.

In order to delve further into elucidating the mechanism of resistance, it is necessary to analyze changes induced at the proteomic level. Two-dimensional electrophoresis and mass spectrometry have previously been used to study proteome-level changes in pea roots and *B. napus* under biotic and abiotic stresses (Kav et al. 2004; Srivastava et al. 2004; Liang et al. 2008). However, to the best of our knowledge this study is the first of its kind to examine proteome-level changes as a result of insect herbivory. Mass spectrometry analysis identified 33 proteins that underwent statistically significant fold changes including cysteine proteases and protease inhibitors (Table 1).

Cysteine proteases are involved with a variety of cellular functions including apoptosis and have been known to play a role in herbivory defense (Pechan et al. 2002; Mohan et al. 2006). Maize insect resistance 1-cysteine protease (Mir1-CP) from insect-resistant maize was shown to disrupt the peritrophic membrane within the insect midgut, interfering with growth and development (Pechan et al. 2002; Lopez et al. 2007). Most interesting was the fact that the identified cysteine protease was abundant in two times the amount of damaged ABR17 when

compared with undamaged seedlings (Table 1). This confirms previous studies that have utilized cysteine proteases to confer insect resistance. Further studies would have to be conducted in order to verify other mechanisms of resistance but it is logical to assume that the increased abundance has a significant impact on *P. cruciferae* resistance.

A number of other metabolic proteins were identified as having increased abundance in plants that experienced flea beetle feeding damage. Previous studies have identified a number of metabolic enzymes that increase in expression in *B. napus* when exposed to pathogen and abiotic stress conditions (Sharma et al. 2006; Srivastava et al. 2007). Interestingly, the Bet V family of proteins is responsible for allergic reactions to birch pollen (Mogensen et al. 2007; Buters et al. 2010). Our results demonstrated higher levels of expression for the Bet v protein (Table 1) in undamaged samples. Similar to the cysteine proteases identified earlier, Bet v 1 may also play a role in selective feeding habits of flea beetles. It is important to note that observed changes in the proteome may not be exclusively caused by herbivory damage. The shot-holes caused by flea beetle feeding may also result in dehydration which itself is a form of abiotic stress.

Many individuals in society today are extremely wary and distrustful of genetically modified organisms (GMOs). To date, there have been few long-term studies investigating the effects of GMOs on the health of the environment and plant and animal populations. A quick internet search will demonstrate the extreme polarities of views on this subject (Global Research 2009; GMO Compass 2010). Until results of a number of objective, independent scientific studies are released that show that ecological and human safety are maintained during the long-term use of GMOs, there will always be apprehension in relation to GMO adoption. Some industries are geared towards generating economic wealth at the expense of protecting the public's best interest, so to see such differences in views from the general population and

those responsible for developing GMOs is no surprise. It is our duty as researchers and academic scholars to ensure that our research is geared toward the discovery of scientific truth with a long-term goal of improving human well-being. It would be extremely rash to push forward, for instance, with adopting transgenic canola like ABR17 without performing the most comprehensive studies to ensure ecological and human safety.

This study has introduced interesting new directions of research that can be pursued in the future. Gene pyramiding utilizes the expression of two or more toxin genes within a single variety (Bates et al. 2005). For instance, by expressing two different insect toxins with distinct binding sites, the possibility of encountering insect individuals resistant to both is greatly reduced, although some potential still exists. Employing refuge zones is also a strategy that can be used to protect the novel germplasm. Incorporating a mixture of resistant and non-resistant plants within the field can help to ensure that resistant alleles in the insects are diluted by random mating with insects with non-resistant alleles. This of course assumes random mating habits and requires management. However, host plant resistance should be used in combination with other cultural and biological strategies for optimal insect pest management (Dent 1990). Classical biological control of these pests has been attempted through introduction of parasitoids from the native host range of flea beetles, but with minimal success (Dosdall and Mason 2010). In addition to the cultural control strategies already evaluated and recommended (e.g., Dosdall et al. 1999; Elliott et al. 2007; Dosdall and Stevenson 2005; Hummel et al. 2009), another strategy for flea beetle control that does not yet appear to have been evaluated includes assessment of push-pull strategies. A push-pull system for insect pest management functions by seeding an attractive, susceptible crop on the outer edge of the main cash crop; the main crop within the susceptible crop possesses properties to repel or deter herbivores (Cook et al. 2007). In canola cropping systems, a push-pull system for flea beetles could utilize an outer

crop of *Brassica rapa* L., which is quite susceptible to flea beetle attack (Dosdall et al. 1999). With a main crop of transgenic germplasm derived from ABR17, which we have shown to be antixenotic and causes a non-preference response in *P. cruciferae*, the potential exists for the beetles to be concentrated in the *B. rapa* perimeter. The perimeter can then be sprayed or plowed under, effectively managing the pest population. Adopting a push-pull strategy may not be attractive to farmers since it would incur a financial loss at the extent of the cash crop, but introducing government subsidies to account for these losses would be a much more attractive method for ensuring its success.

Transgenic ABR17 and PR10.1 represent a great potential for reducing the chemical insecticides that enter our agroecosystems. The fact that the ABR17 gene has been shown to be completely viable when expressed in peas, canola and *A. thaliana* opens the door to testing how it will perform against insect pests in other plant species. Our results are new and exciting and represent the first application of PR proteins for reducing insect pest damage. Future studies will require field testing in order to discover how ABR17 and PR10.1 perform in ambient, field conditions. Long-term studies will need to be conducted to determine how the transgenic plants will affect the environment and whether or not they possess any risk to humans. Elements in our society are already wary and distrustful of GMOs and they must be assured of product safety. By educating the farmers on how to best implement strategies to increase their yields, the longevity of the novel germplasms and preserve the environment we can create a holistic approach to managing the world's increasing food crisis. As the global population grows beyond what our arable land can sustain, it is becoming more and more important to turn to techniques that can use the land more efficiently.

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