Analysis of *CYP81F2-A10*, a potential candidate gene for the broad spectrum defense to *Leptosphaeria maculans* in *Brassica napus* L.

by

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Foreword

This thesis follows the manuscript style outlined by the Department of Plant Science and the Faculty of Graduate Studies at the University of Manitoba. The manuscript follows the style of the Canadian Journal of Plant Science. Presented as a single manuscript, this thesis contains an abstract, introduction, materials and methods, results, and a discussion. Literature review is presented immediately before the manuscript and conclusions follow the end of the manuscript. Supplementary tables and figures follow immediately after the conclusion.

Abstract

Canola (*Brassica napus* L.) is an economically important crop for producers in Canada and the ability to reduce disease causing yield loss is necessary for the longevity of the crop. A major disease that impacts canola production is blackleg, caused by the fungal pathogen *Leptosphaeria maculans* (Desm.) Ces. Et de Not. The understanding of how both quantitative and qualitative resistance to this disease works is vital to disease management. In this thesis, the response of the candidate gene *CYP81F2-A10* (homolog of the A*rabidopsis thaliana* (L.) Heynh gene CYP81F2), and the 4-methoxy-indole-3yl-methyl-glucosinolate concentration upon infection examined using three *B. napus* near-isogenic lines (NILs) of Westar containing the allele *CYP81F2-A10_Surpass400*, and wild type, Westar containing the allele *CYP81F2-A10_Westar*. Plants were inoculated with either *L. maculans* (Isolate 87-41) or mock control (H_2O). Relative to Westar, the NILs showed the first sign of suppression in pathogen growth and development at 6 days post inoculation (DPI). Full cDNA sequencing of *CYP81F2-A10* revealed that *CYP81F2-A10_Surpass400* contained a singular point mutation, resulting in an amino acid change. Analyses of transcript levels by RT-qPCR revealed that all lines showed upregulation of *CYP81F2-A10* upon inoculation with *L. maculans.* The NILs showed gradual upregulation of the 4-methoxy-indole-3-yl-methyl-glucosinolate in response to inoculation with *L. maculans*, and two NILs (HN7 and NJ11) showed a significant difference from both their mock control treatment and inoculated Westar at 6 DPI. Westar did not show the same gradual increase in 4-methoxy-indole-3-yl-methyl-glucosinolate until 8DPI, where this increase did not correspond with pathogen suppression. This study suggests that the candidate gene *CYP81F2-A10* is involved in the upregulation in 4-methoxy-indole-3-yl-methyl-glucosinolate in response to *L. maculans* inoculation in the early stages of

infection. Furthermore, this type of interaction is associated with broad spectrum resistance and is non-specific to *Avr* genes.

1.0 Literature Review

1.1 Host Background

1.1.1 Brassica Species

The *Brassicaceae* family is composed of over 4000 species from approximately 300 genera and includes a diverse population of plants (Warwick et al. 2006). Within this family there are three primary species, *Brassica rapa* L. (2*n*= 20, AA), *Brassica nigra* (L.) W.D.J. Koch (2*n*=16, BB), and *Brassica oleracea* L. (2*n*=18, CC), and three secondary amphidiploids derived from natural hybridization and genome doubling: *Brassica juncea* (L.) Czern (2*n*=36, AABB), *Brassica carinata* A. Braun (2*n*=34, BBCC), and *Brassica napus* L. (2*n*=38, AACC) (Figure 1.1) (Nagaharu 1935; Warwick 2011; Friedt et al. 2018).

1.1.2 History of *Brassica napus*

Brassica napus is an allotetraploid species belonging to the Cruciferae family, also referred to as oilseed rape (Warwick 2011; Sharma et al. 2014). *Brassica napus* belongs to the mustard family and is the result of hybridization between *B. rapa* and *B. oleracea* about 7500 years ago (Chalhoub et al. 2014; Sharma et al. 2014; Rahman et al. 2018). The rise in *B. napus* hectares in North America began due to lubrication demand during World War II (Boulter 1983; Barthet 2015). This increase in growth of *B. napus* was successful and suggested that much of North America had an ideal growing environment for its production (Eskin and Przybylski 2003). Experts saw a gap in local production for edible oils that could potentially be filled with *B. napus*; however, cultivars present at the time contained high levels of erucic acid and glucosinolates in the meal (Eskin and Przybylski 2003). These components generate an undesirable flavor and fatty acid profile that are not ideal in large quantities for an edible oil (Barthet 2015).

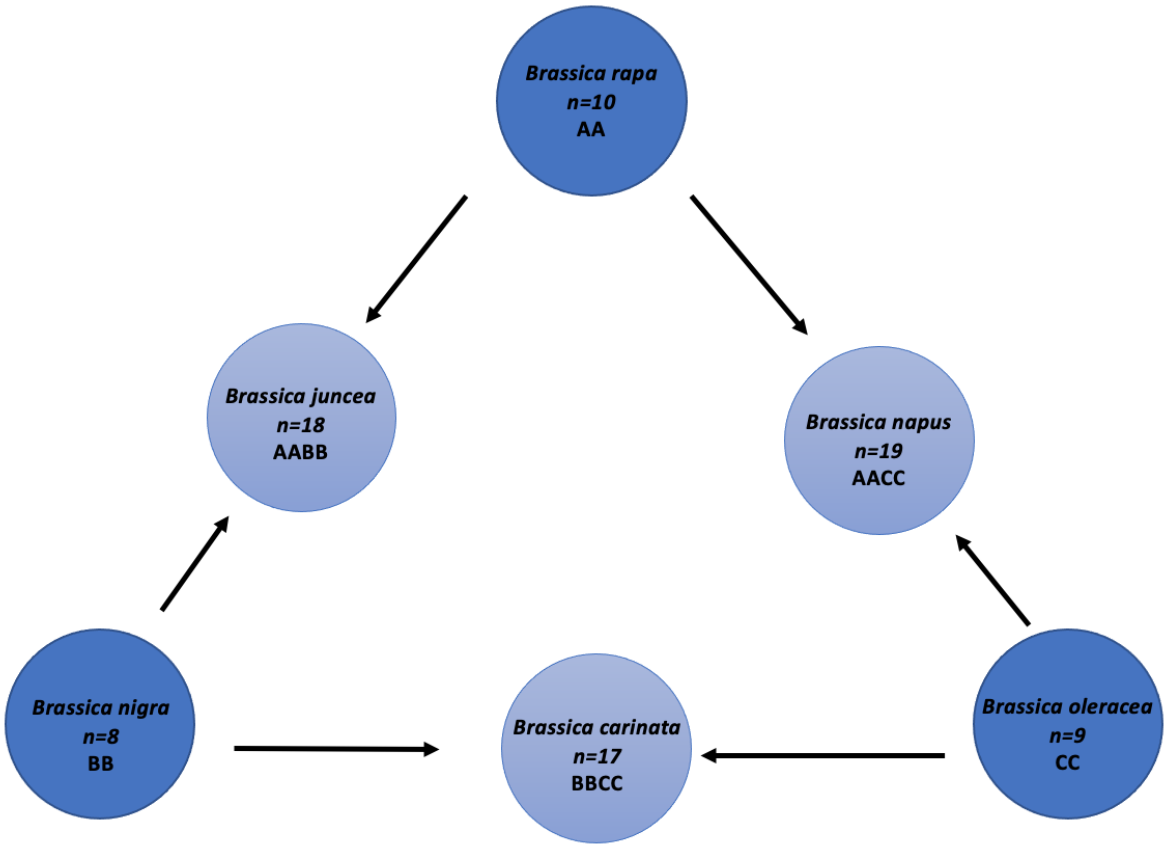

Figure 1.1 The "Triangle of U" representing the relationship of the three primary and three secondary Brassica species. Adapted from (Nagaharu 1935).

By 1974, researchers successfully integrated low erucic acid and low glucosinolate characteristics into a single cultivar, and the first “canola” quality cultivar, ‘Tower’, was registered (Stefansson and Kondra 1975). Canola is a term that was trademarked to define the new edible oil developed from *Brassica* species. The term canola comes from Can (Canada) and ola (oil) and defines the nutritional profile of the plant (Canola Council of Canada 2021). Currently, the standards for canola require less than 2% erucic acid and less than 30 micromoles of glucosinolates per gram (Canola Council of Canada 2021).

Canola production within Canada has had a substantial impact on the economy, with 8.7 million hectares grown in 2022, and has seen rapid growth over the past decade (Statistics Canada 2022). Canola’s contribution to the Canadian economy has tripled over the last decade, standing at over $29.9 billion annually and generating an estimated 207,000 jobs (Canola Council of Canada 2023a).

1.1.3 Production and Usage of Canola

Production of canola requires fertile, well drained soils, with a firm fine textured seed bed, and low number of weeds and surface residue (Raymer et al. 1990). It is recommended to plant in early spring (May 1st-June 20th) in Canada, at a seeding depth of 1.5-3.0 cm, into soil with minimum or zero till to conserve soil moisture (Raymer et al. 1990; Kharbanda and Tewari 1996; Manitoba Agriculture 2007). Canola removes around 68-83 kg/ha nitrogen, 37-45 kg/ha phosphate, 18-22 kg/ha potassium, and 11-13 kg/ha sulfur (Manitoba Agriculture 2007). Fertilizer requirements can vary depending on the previous crop and current soil fertility (Manitoba Agriculture 2007). Canola can be direct harvested or swathed, and seed moisture must be 10 % or less for direct harvest and 35-40 % for swathing. Further dry down to 8 % or less moisture content is required for proper storage (Manitoba Agriculture 2007; Canola Council of Canada 2023b).

The demand for canola depends on two main end-product uses, oil and meal, as well as seed production (Daun 2011; Unger 2011). End-use products are the result of crushing, extraction, and refining of the oil, which also results in the meal by-product (Unger 2011). The oil produced can be utilized as edible oil, or for fine chemicals, efficient fuels, and lubricants, depending on the oil composition (Daun 2011). Canola meal, which is a by-product of oil production, has been used as a high-quality animal feed, a functional protein source, and a fertilizer (Daun 2011).

1.2 Blackleg Disease

A significant disease impacting canola production is blackleg, caused by fungal pathogens from the *Leptospheria* genus, comprising of a more aggressive species *Leptospheria maculans* (Des.) Ces. Et de Not. (Tulasne and Tulasne 1863), and a weakly virulent species *Leptosphaeria biglobosa* n.sp. (Shoemaker and Brun 2001). The first significant cases of blackleg infection were reported in the 1970s in Saskatchewan (Gugel and Petrie 1992), and by the 1980s blackleg had become a recurrent issue for canola production across Canada (Kutcher et al. 2011).

1.2.1 Disease Cycle

The pathogen causing blackleg has both a sexual and asexual mode of reproduction and can produce both primary and secondary inoculum (Hall 1992). While ascospores, pycnidiospores, and mycelium are all forms of primary inoculum, in Canada, ascospores are the most important primary source and pycnidiospores are the main source of secondary inoculum through asexual reproduction (Figure 1.2) (Hall 1992; Ash 2000; Guo and Fernando 2005). These sources are mainly derived from infected canola and Brassica species stubble (Hall 1992). Infected seed can

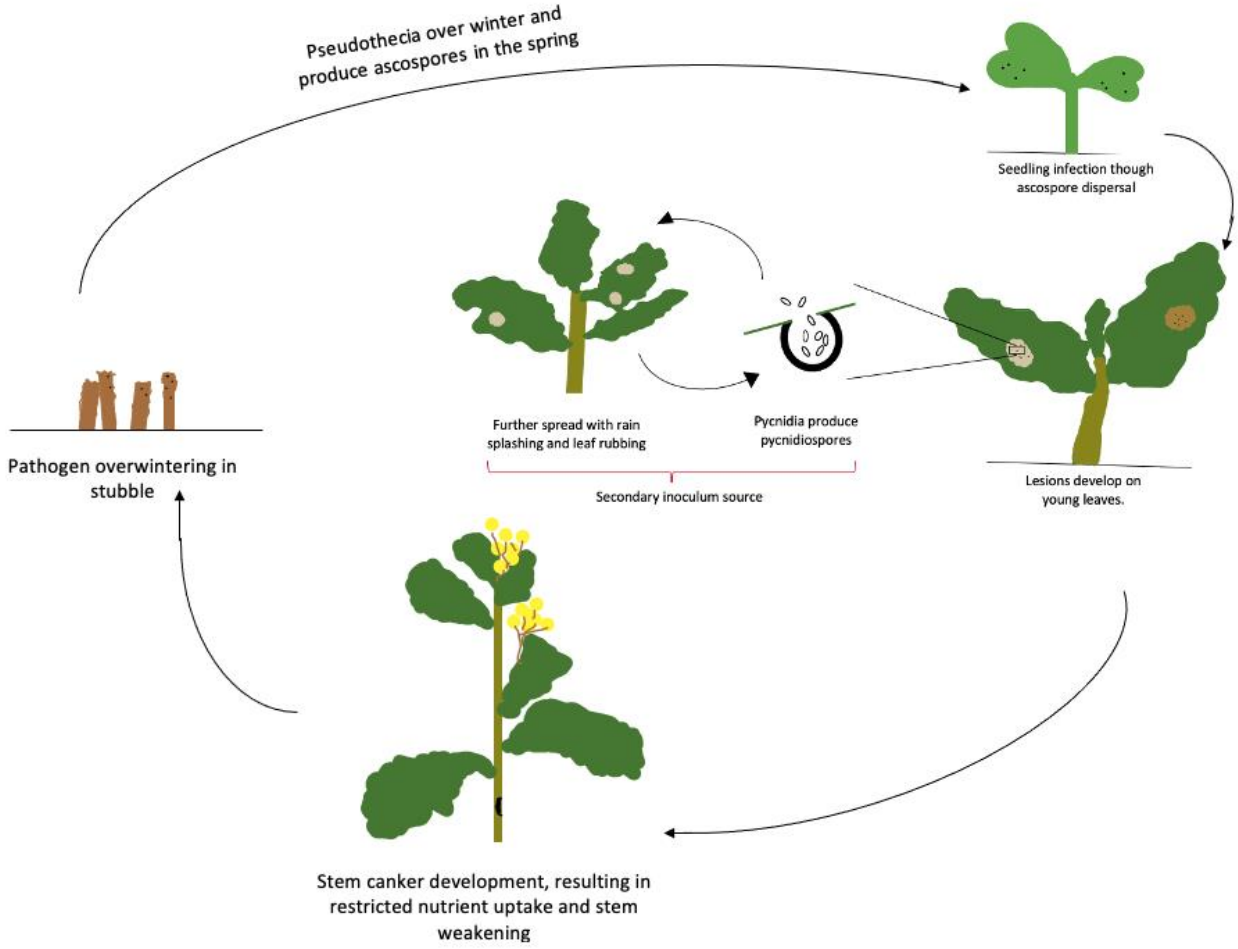

Figure 1.2. Life cycle of *Leptosphaeria maculans* in canola in western Canada. Adapted from (Ash 2000).

also be a source of infection and can spread over large geographical regions (Gugel and Petrie 1992). However, due to the already present establishment of blackleg in most areas, wind dispersal is a much more significant factor in the spread of *L. maculans* compared to infected seed (Gugel and Petrie 1992).

Sexual reproduction of *L. maculans* is key for the overwintering on infected stubble in the form of pseudothecia on stem tissue, or mycelium (Rouxel and Balesdent 2005). Pseudothecia can produce and release ascospores at temperatures as low as 8-12 °C and therefore result in ascospores being released in the early springtime when plants begin emergence and are vulnerable (Hall 1992; Rouxel and Balesdent 2005). Ascospores are generally released during rainfall or wind and can be dispersed up to 10 km away, making control of spread very difficult due to long-range dispersal (Ash 2000; Rouxel and Balesdent 2005; Fernando et al. 2007). Ascospores land on cotyledons or basal leaves and begin infection through stomata or tissue wounds, growing and colonizing through intercellular spaces between mesophyll cells (Fernando et al. 2007). This results in grey/brown leaf lesions, the first visual sign of infection (Ash 2000). Beneath the leaf surface, the fungus invades and colonizes both leaf and stem tissue but remains visually symptomless on the exterior for an extended period (Williams 1992). In adult plants, the fungus destroys crown tissue, causing the stem canker and base stem weakening, which can further result in plant lodging and yield loss). The pseudothecia will survive overwinter in infected stubble (Rouxel and Balesdent 2005).

Asexual reproduction is the main cause of secondary infection (Ash 2000). Asexual spores called pycnidiospores develop initially from infected leaf lesion sites and are not readily airborne; they are usually released in the form of mucilage to facilitate spread via rain splashes and rubbing of nearby plants (Ash 2000; Fernando et al. 2007). While pycnidiospores do overwinter, their slow

germination rate, stricter environmental conditions, and limited spread make them a minor aspect of the primary disease. These factors make secondary infection less likely to cause major yield loss in Canada (due to cankers) (Ash 2000).

In terms of host susceptibility, the growth stage of the plant plays a big role in the ability of the pathogen to infect and cause disease (Shahoveisi et al. 2022). *Leptosphaeria maculans* enters leaves and cotyledons from the stomata or wounds and then infects tissues downward until it has reached the base of the stem (Marcroft 2019). Fully developed plants are more resistant to infection and disease symptoms because the pathogen must travel a longer distance to reach the base of the stem (Shahoveisi et al. 2022). This is in contrast to seedlings where the establishment of the pathogen is faster (Marcroft 2019).

1.2.2 Symptomology

The initial infection is associated with white to tan-colored lesions of necrotic tissue that are irregular or round-shaped and have small spots of black pycnidia on the cotyledons or true leaves (Guo and Fernando 2005; Canola Council of Canada 2020). Once the initial pathogen infection takes place, the plant is relatively symptomless until the fungal pathogen destroys the crown tissue and causes the stem canker (the most recognized symptom of blackleg) (Rouxel and Balesdent 2005). Stem cankers appear near the end of the growing season in adult plants and are dark grey/black with brown margins near the base of the stem and can be sunken. This can result in lodging due to stem weakening (Fernando et al. 2007). Stem cankers may not be seen on the outside of the plant if the severity of infection is moderate, but a cross-section of the stem will reveal black necrotic tissue that restricts nutrient and water uptake, resulting in decreased yield (Canola Council of Canada 2020).

In 2016, upper canopy infection from asexual *L. maculans* spore production was identified and is becoming an increasing problem for yield loss in Australia (Sprague et al. 2018; Marcroft 2019). Upper canopy infection presents as blistering of the upper canopy stems (showing large tan lesions with black spores), abortion of flower heads, pod abortion, and necrosis of the branch pith (Sprague et al. 2018). Upper canopy infection is rarely seen in Canada due to the vastly different cultural practices and climate relative to Australia (Ie singular harvest per year, dryer conditions at harvest, ascospores germinating in spring)(Fitt et al. 2006; Kutcher et al. 2010; Marcroft 2019).

1.2.3 Environmental Conditions Promoting Disease Spread

Due to characteristics in the blackleg life cycle, some environmental conditions are favorable for disease infection and spread. These conditions include early rainfall and wind which disperse conidia and ascospores (Williams 1992), and late-season crops which are not established and are more vulnerable when the fungus begins to infect (Gugel and Petrie 1992). Laboratory experiments showed ascospores germinated in a little as 4 hours at temperatures between 4 and 28 °C; this finding suggests that wetness is the limiting factor in infection rather than temperature (Hall 1992; West et al. 2001). This is further demonstrated by the delay seen in pseudothecial maturity in western Australia and in Canada versus the rapid maturation of pseudothecia seen in the UK (in some cases can be seen shortly after harvest) (West et al. 1999; Toscano-Underwood et al. 2003). This delay is due to dry hot summers and subzero winter temperatures of western Australia and Canada, respectively. Both of these conditions limit water availability, compared to the mild wet weather conditions in UK (Toscano-Underwood et al. 2003). Reducing relative humidity from 70% to 40-50% decreased the aggressiveness in *L. maculans* (El Hadrami et al. 2010). However, this study looked at constant relative humidity in a greenhouse setting, and further research into how humidity affects disease at a field scale needs to be conducted (El Hadrami et al. 2010).

1.2.4 Pathogen

Leptosphaeria maculans is a hemibiotrophic pathogen affecting many cruciferous crops (Bokor et al. 1975). Originally, 4 major pathogenicity groups (PG 1-4) were described (Mengistu et al. 1991). This original classification system was based on the degree of reaction of infected cotyledons among Westar, Glacier, and Quinta (Mengistu et al. 1991). Pathogenicity group 1 denotes lack of virulence and PG-4 denotes complete virulence (Mengistu et al. 1991). Pathogenicity group 1 includes the less virulent species *L. biglobosa*, while the highly virulent more common species of *L. maculans* belong to PG-2, 3, and 4 (Chen and Fernando 2006). Species with the PG1 distinction display an avirulent interaction on all 3 genotypes, PG2 displays virulence on Westar but avirulence on Glacier and Quinta, PG3 displays virulence on Westar and Glacier and avirulence on Quinta, while PG4 displays virulence on all three genotypes (Chen and Fernando 2006). This classification system does not allow for the identification of genetic variability within the pathogen population, but rather only identifies if the isolates are susceptible or resistant to these specific host genotypes (Fernando et al. 2007; Kutcher and Yu 2009).

Due to the large variability in the pathogen population, there was a need to identify more specifically the genetic interaction. This need led to further classification of the *Avr* genes within *L. maculans* (Balesdent et al. 2005; Delourme et al. 2006). Pathogen isolates can carry one or more *Avr* genes, and to date 17 *Avr* genes have been identified (Table 1.1). The identification of these *Avr* genes allows for targeting specific virulent pathogen isolates in a particular region for resistance purpose (Kutcher and Yu 2009).

Table 1.1. Major resistance genes in *Brassica napus* L. for resistance to *Leptospheria maculans* and the corresponding *avirulence* genes.

Major Resistance Gene	Avirulence gene	Reference

Rlm1	*AvrLm1*	(Ansan-Melayah et al. 1995; Delourme et al. 2004)
Rlm2	*AvrLm2*	(Ansan-Melayah et al. 1995; Delourme et al. 2004; Ghanbarnia et al. 2015)
Rlm3	*AvrLm3*	(Balesdent et al. 2002; Delourme et al. 2004)
Rlm4	*AvrLm4-7*	(Ferreira et al. 1995; Rouxel et al. 2003; Tollenaere et al. 2012)
Rlm5	*AvrLm5-9*	(Balesdent et al. 2002; Plissonneau et al. 2018)
Rlm6	*AvrLm6*	(Chèvre et al. 1996; Balesdent et al. 2002; Fudal et al. 2007; Brun et al. 2010)
Rml7	*AvrLm4-7*	(Balesdent et al. 2002; Delourme et al. 2004)
Rlm8	*AvrLm8*	(Delourme et al. 2004)
Rlm9	*AvrLm5-9*	(Balesdent et al. 2002; Delourme et al. 2004; Ghanbarnia et al. 2018)
Rlm10	*AvrLm10a* *AvrLm10b*	(Eber et al. 2011; Petit-Houdenot et al. 2019)
Rlm11	*AvrLm11*	(Balesdent et al. 2013)
LepR1	*AvrLepR1*	(Yu et al. 2005)
LepR2	*AvrLepR2*	(Yu et al. 2005)
LepR3	*AvrLepR3*	(Ansan-Melayah et al. 1998; Larkan et al. 2013)
LepR4a *LepR4b*	*AvrLepR4*	(Cantila et al. 2021)
RlmS-	*AvrLmS-*	(Xiang Neik et al. 2022)

1.2.5 Disease Management

Due to the nature of *L. maculans*, there is not a single specific factor that offers complete disease control in canola (Gugel and Petrie 1992; Delourme et al. 2006). While genetic resistance is

extremely effective when there is an incompatible interaction, the increase in virulent isolates and the frequency of resistance breakdown suggests that blackleg management needs to be an integrated approach comprised of multiple strategies (Gugel and Petrie 1992; Delourme et al. 2006). In Canada, chemical and biological control can be used to reduce the incidence and severity of blackleg; however, cultural and genetic control are far more popular and widely used (Gugel and Petrie 1992; Zhang and Fernando 2018).

1.2.3.1 Chemical Management

Chemical management in the form of foliar fungicides or seed treatments has been developed for blackleg control and can be effective in some situations (Hwang et al. 2016; Canola Council of Canada 2023c). However, chemical management of blackleg in Canada has not been a popular avenue. Of the main reasons foliar fungicides are an unpopular strategy is due to the nature in which the fungicides work. They have little to no eradicant activity and are needed to be applied before symptoms appear (Ulrich et al. 2000; Peng et al. 2021). Additionally, studies have determined that the effectiveness of fungicide in reducing disease or increasing yield is highly dependent on many factors like the environment, pathogen and cultivar (Ulrich et al. 2000; Fraser et al. 2020). When the use of fungicides did reduce pathogen incidence and increase yield it was only seen on susceptible and moderately resistant cultivars (Ulrich et al. 2000; Fraser et al. 2020; Peng et al. 2021). With the availability of disease resistant cultivars and the inconsistent impact on in yield, chemical management has been an unpopular method among producers (Canola Council of Canada 2023c).

1.2.3.2 Biological Control

Biological control can be defined as “the reduction of inoculum density or disease-producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms,

accomplished naturally or through manipulation of the environment, host, or antagonist, or by mass introduction of one or more antagonists" (Baker and Cook 1974). Due to the nature of *L. maculans,* there are several possible mechanisms through which biological control can be applied including the use of weakly-virulent pathotypes, competing pathogens, management of stubble, and soil bacteria (West et al. 2001; Pedras et al. 2003; Chen and Fernando 2006; Fernando et al. 2007). To date, there has been no effective and consistent agent that has proven to be suitable for commercial use in the management of *L. maculans* on canola (Fernando et al. 2007).

There have been studies for both the inoculation of weakly-virulent strains of *L. maculans* and inoculation of *L. biglobosa,* to induce suppression of virulent pathotypes for biological control (Mahuku et al. 1996; Chen and Fernando 2006; Fernando et al. 2007). This research has shown some positive indications by hypersensitive response and systematic acquired resistance, respectively (Mahuku et al. 1996; Chen and Fernando 2006; Fernando et al. 2007).

The use of competing pathogens in reducing or eliminating *L. maculans* includes *Pseudomonas fluorescens* Migual and *Paenibacillus polymyxa* Prazmowski, both of which demonstrated antifungal activity against *L. maculans* (West et al. 2001; Pedras et al. 2003). However, the cost to deploy competing pathogens, both economically and agronomically, is likely too high for the associated benefit and needs to be further explored (Smith et al. 2008; Canola Council of Canada 2023c).

Infected stubble is a major cause of reinfection in *L. maculans,* and management of this stubble can have major impacts on incidence of infection (West et al. 2001). Studies on two species of birds nest fungus, *Cyathus striatus* (Huds.) Willd, and *C. olla* (Batsch) Persoon, have found that their ability to feed on stubble can help to further reduce the buildup of *L. maculans* inoculum

during field rotations (West et al. 2001). Finally, a few select bacterial strains (*Pseudomonas* (Migual) and *Bacillus* (Cohn)) identified within the rhizosphere of canola have been shown to be strong blackleg suppressors, reducing the overall infected stubble (Fernando et al. 2007). Further research is needed to explore the mechanism of action within these systems to validate the biological control of infected stubble (Fernando et al. 2007).

Due to the requirements for biological control, and the associated high costs, at the moment there is no identified form of biocontrol for *L. maculans* that can be widely applied in commercial production. Any biological control agent needs to have consistent and reliable results (potentially as an eradicant), not harm the environment, and be cost effective (Fernando et al. 2007; Cornelsen 2017; Canola Council of Canada 2023c).

1.2.3.3 Cultural Management

Cultural management of disease refers to the activities of producers conducted to manage disease through the cultural manipulation of plants (Ogle and Dale 1997). Gugel and Petrie (1992) stated that management of plant diseases is achieved by disrupting the sequence of events necessary for the disease to establish infection. Cultural management achieves this through practices like destruction of crop residue, weed management, tillage practices, planting and harvesting practices, nutrient management, and many other practices depending on the crop and disease (Ogle and Dale 1997).

Cultural practice is an important factor in all disease management, and this notion remains true for *L. maculans* (Palm 1968; Walters 2009). In the case of blackleg, infected stubble represents a key factor in disease spread (Gugel and Petrie 1992). Tight rotations and close proximity of newly sown fields with previous stubble can promote disease spread (Gugel and Petrie 1992). Some studies on rapeseed in Europe have shown that early planting and limiting nitrogen availability

have reduced incidence and severity of crown stem canker caused by *L. maculans* (Aubertot et al. 2004). In Canada, the most popular cultural management practice is crop rotation, with the recommendation of a four-year break between *Brassica* cultivars (West et al. 2001). Additionally, long range wind dispersal of spores has been observed and it is recommended to plant canola crops at minimum 50 to 100 meters from each other and from the previous year's canola fields (Guo and Fernando 2005). In the past, it was believed that removing stubble as much as possible, through tillage and burning, represented the best management (Marcroft and Potter 2007). However, several studies have disproved this practice (Marcroft and Potter 2007). Bushfires in southern Australia failed to show significantly reduced incidence of blackleg infection in crops after the burns (Marcroft and Potter 2007). Given the indirect consequences of burning (air pollution, and soil microbe damage), these stubble management practices are not recommended (Gugel and Petrie 1992; Marcroft and Potter 2007; Canola Council of Canada 2023c).

1.2.3.4 Genetic Resistance

The use of cultivar resistance is the most common strategy for blackleg disease control (Kutcher et al. 2011). Over the past few years, the understanding of the plant responses to cope with *L. maculans* has greatly improved, and two mechanisms have been identified: qualitative and quantitative (Flor 1971; Sprague et al. 2018; Cornelsen et al. 2021). Qualitative resistance derives from major allelic differences at one or two genes and results in extremes in resistance (Kearsey and Pooni 1996; Poland et al. 2009). Quantitative resistance derives from few to many genes interacting with the environment and each other, and results in phenotypic variance (Kearsey and Pooni 1996; Mackay 2001; St.Clair 2010). In disease resistance, quantitative resistance often displays a reduction in disease severity rather than an absence of disease (Young 1996; Poland et al. 2009; St.Clair 2010). Quantitative resistance is much more complex and understanding the

methods in which these genes interact and how to integrate them into cultivars for commercialization is often poorly understood (Sprague et al. 2018). Thus, in canola, only qualitative resistance is currently available in commercialized cultivars for blackleg resistance (Sprague et al. 2018).

Qualitative resistance of blackleg in canola is often referred to as gene-for-gene theory in which the specific *Avr* genes of *L. maculans* are recognized by the resistance genes contained within the cultivar (eg *Rlm1*|*AvrLm1*) (Flor 1956; Delourme et al. 2006). In this case, there is complete resistance to the specific pathogen isolate (Delourme et al. 2006). There are two possible interactions in this system: compatible, where there is no host defense response due to a lack of resistance genes or a lack of corresponding *Avr* genes, and incompatible, where the host defense and the *Avr* genes interact to prevent disease development (Raman et al. 2013). As mentioned previously, initial studies classified *L. maculans* into Pathogenicity Groups (PG) based on their avirulence on several *B.napus* cultivars such as 'Westar', 'Quinta', and 'Glacier' (Delourme et al. 2006). Later 'Lirabon', 'Quinta', 'Glacier', and 'Jet Neuf' were used to further identify the differences in the PGs by splitting the groups based on the initial pathogenicity characteristics and then further by their virulence or avirulence to 'Jet Neuf'; this resulted in six PG termed A1-A6 (Delourme et al. 2006). Later on, it was suggested that race specific characterization of *L. maculans* would be more effective (Balesdent et al. 2005). This type of characterization requires cotyledon tests and individual isolates of *L. maculans* to distinguish the *Avr*-R gene interactions (Alnajar et al. 2022). Initial studies identified 5 R genes contained in the brassica genome; *Rlm1, Rlm3, Rlm4, Rlm7,* and *Rlm9* (Delourme et al. 2006). Further studies identified 12 additional resistance genes for a total of 17 major resistance genes (Table1.1). The use of the avirulent allele classification for *L. maculans,* vs. the original pathogenicity group classification, allows for the identification of the

avirulent allele frequency within an area, and targets the specific alleles with specific resistance genes, resulting in a more accurate resistance outcome (Kutcher et al. 2010). Cultivars are classified by their field resistance labels (R= Resistance, MR= Moderate Resistance, MS= Moderate Susceptible, S= Susceptible) followed by the resistance group (Delourme et al. 2006; Cornelsen 2017).

1.2.5.4.1 Resistance breakdown

Genetic resistance is only useful in commercial cultivation if it is durable, meaning it needs to remain effective over long periods in the disease favorable environment (Brun et al. 2010). The use of R-gene-mediated resistance (qualitative resistance) is associated with area-specific isolates which provide complete resistance, and exhibit a strong selection pressure, promoting the population to adapt and shift due to selection and reduced competition for virulent pathogens (Brun et al. 2010). In *B. napus*, this resistance breakdown can happen in a short period of time. In the case of the cultivar 'Surpass 400' (which contains the *Rlm1* gene), it exhibited a high level of adult plant resistance, but this only lasted three years before being deemed as inefficient in South Australia (Sprague et al. 2006). This is largely due to the typical life cycle of *L. maculans*, that can produce a large number of spores that are spread long distances via wind, as well as localized spores spread by rain splash (Sprague et al. 2006). In addition, the pathogen goes through an annual sexual lifecycle and has asexual spore production, all characteristics that support larger evolutionary potential (McDonald and Linde 2002; Sprague et al. 2006).

The constant use of single R genes in tight rotations places extreme selection pressure on the pathogen population (Brun et al. 2010). This extreme selection pressure promotes resistance breakdown by artificially inflating the natural selection that occurs. This creates opportunities for new pathogen diversity generated through mutation and recombination (Strelkov and Canola

Council of Canada 2018). This then causes a population shift around the specific resistance genes, rendering the R genes ineffective (also known as R gene breakdown) (Brun et al. 2010).

1.3 The Potential of Quantitative Resistance

Quantitative resistance refers to a polygenic development of resistance, where incomplete or partial resistance of a phenotype is often seen (Niks et al. 2015; Corwin and Kliebenstein 2017; Pilet-Nayel et al. 2017). This type of resistance in plant/pathogen interactions does not completely block pathogen growth and development but reduces many aspects vital to pathogen infection like multiplication, colonization, and symptom severity (Pilet-Nayel et al. 2017). While this type of resistance is often referred to as partial resistance, a combination of multiple quantitative genes can provide strong effects to confer high levels of resistance (Niks et al. 2015; Pilet-Nayel et al. 2017). This is the case for bacterial spot pathogen in *Solanum lycopersicum* L., where there was a high level of resistance to *Xanthomonas campestris* pv. *vesicatoria* (Doidge) in a Hawaiian tomato accession that had a varying level of resistance in the F_2 population (Stall et al. 2009).

Suggestions to improve resistance in the canola/blackleg pathosystem have been proposed by integrating both quantitative and qualitative genes to provide added protection (Brun et al. 2010). However, quantitative resistance genes have proven to be both biologically and technically complex to identify. To mitigate this, it was proposed to integrate major resistance genes into cultivars exhibiting a high level of quantitative resistance (Brun et al. 2010; Corwin and Kliebenstein 2017). Additionally, quantitative resistance alone in commercial production was found to be only partially effective because favorable environmental and plant tissue conditions for disease left the plant vulnerable (Somda et al. 1999; Brun et al. 2000). By integrating both quantitative and qualitative resistance genes there is the potential to improve the durability of R-

gene-mediated resistance (Brun et al. 2010). In theory, quantitative resistance should limit the spread and rate the pathogen evolves (Palloix et al. 2009; Brun et al. 2010).

While the integration of R genes into cultivars that displayed high levels of quantitative resistance has had some success (Brun et al. 2010), it is necessary to better understand these systems to effectively integrate quantitative resistance into breeding programs (Long et al. 2011). Extensive research and identification of genes involved in the plant-pathogen response has led to the identification of independent loci (*BLMR1* and *BLMR2*) which are involved in the plant immune response (Long et al. 2011). *BLMR1* was identified to be the same as *LepR3*, but *BLMR2* functioned in intermediate cotyledon resistance phenotypes and contributed to adult plant resistance in canola cultivars against blackleg (polygenic resistance) (Long et al. 2011; Dandena et al. 2019). Due to this polygenic display of resistance, it is suspected that this gene is a quantitative resistance gene (Corwin and Kliebenstein 2017; Dandena et al. 2019; Zhang et al. 2021). Recent reports indicate redundancy within the naming of *BLMR2*, *RlmS*, and *LepR2* which have been mapped to the same chromosomal region on chromosome A10. It has been theorized that these are the same R genes (Borhan et al. 2022). Resistance gene *LepR2* was found to limit hyphal growth of avirulent isolates but not prevent it, and field isolates displayed a range of resistance labeled intermediate resistance (Xiang Neik et al. 2022). The naming and terminologies used to describe the *L. maculans-B. napus* pathosystem is still in flux, and the full extent of the involvement and similarity of these genes has yet to be determined (Xiang Neik et al. 2022). For the purpose of this research, the gene in question will be referred to as '*BLMR2'*.

The function of *BLMR2* was investigated using a series of near-isogenic lines and comparative physical mapping, as well as pathogen-induced transcriptome (Zhang et al. 2021). This study determined that the candidate gene believed to be involved in this, which will be referred to as

CYP81F2-A10, is a homolog of *CYP81F2 (At5g57220)* in A*rabidopsis thaliana* (L.) Heynh, which catalyzes the modification of indole Glucosinolates (GSLs) to 4-methyoxy-indol-3-ylmethyl GSL (Zhang et al. 2021).

1.4 Glucosinolates

Glucosinolates (GSLs) are plant secondary metabolites derived from amino acids and sugars and are rich in sulfur (Wang et al. 2011). They are generally classified into three major groups, aliphatic, aromatic and indole GSLs, based on the amino acid they originate (methionine, phenylalanine or tyrosine, and tryptophan respectively) (Halkier and Du 1997). Each of these groups can be further distinguished based on the modifications of the secondary side chains (Halkier and Du 1997). Upon mechanical damage, as a result of biotic or abiotic stressors, GSLs are hydrolyzed by myrosinase (Lüthy and Matile 1984). The resulting products are involved in the plant defence mechanisms (Bones and Rossiter 1996). However, due to their negative effects on meal properties (increased pungent odor and bitter taste) plant breeding strategies in canola have sought to reduce total GSL levels (Alexander et al. 2008). To be considered a canola cultivar, products must contain less than 30 micromoles of GSLs per gram of air-dried oil-free meal (Canola Council of Canada 2021).

Glucosinolate synthesis is composed of several steps; the first involves an amino acid chain elongation step and a core structure formation; this is followed by a secondary side-chain modification step (Wang et al. 2011). While the initial events are common to all three types of GSL, the modification step is the most relevant factor that creates differential types of GSL (Wang et al. 2011).

1.4.1 Primary Production

The first step of GSL production is similar among all types of GSLs and they share a common set of enzymes (Bednarek et al. 2009; Ishida et al. 2014). Apart from chain elongation, which only occurs in aromatic GSLs, the process GSLs production comprises the following steps: *CYP79s* converts amino acids to aldoximes, which are then oxidized to the active forms by *CYP83s*, through *C-S lyase* (Ishida et al. 2014). After production of thiohydroximates, the compounds are converted to their final core structure by *S*-glucosyltransferases (UGT74) and sulfotransferases (Ishida et al. 2014) (Figure 1.3). In Figure 1.4 the final primary core structure of indole-3yl-methyl GSL is depicted; this core structure will further be diversified by side chain modification (Bednarek et al. 2009; Pfalz et al. 2009).

1.4.2 Secondary Side Chain Modification

Glucosinolates are further diversified by side-chain modifications, which vary depending on genotypic and environmental factors (Halkier and Du 1997). They can include hydroxylations, methylations, oxidations, and desaturations (Halkier and Du 1997). These modifications are carried out by various loci (GS-OX, GS-AOP, GS-OH, BZO1, CYP81F2, etc.) and examples of modifications are shown in Figure 1.5 (Halkier and Du 1997; Pfalz et al. 2009; Wang et al. 2011; Zhang et al. 2021). Other factors like nuclear-localized regulators and transcription factors can contribute to further modifications (Halkier and Du 1997). Due to the diversity of the secondary side chains, over 100 different GSLs have been identified in *Arabidopsis* (Halkier and Du 1997; Wang et al. 2011). Side chains contribute to the final physicochemical properties and biological

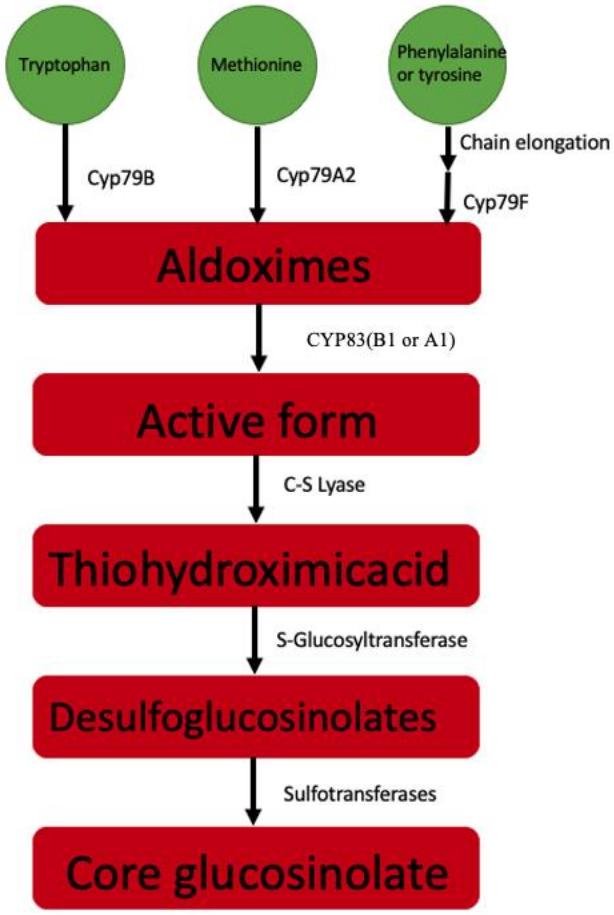

Figure 1.3. Pathway for synthesis of the core structure of glucosinolates. Adapted from Bednarek et al. (2009).

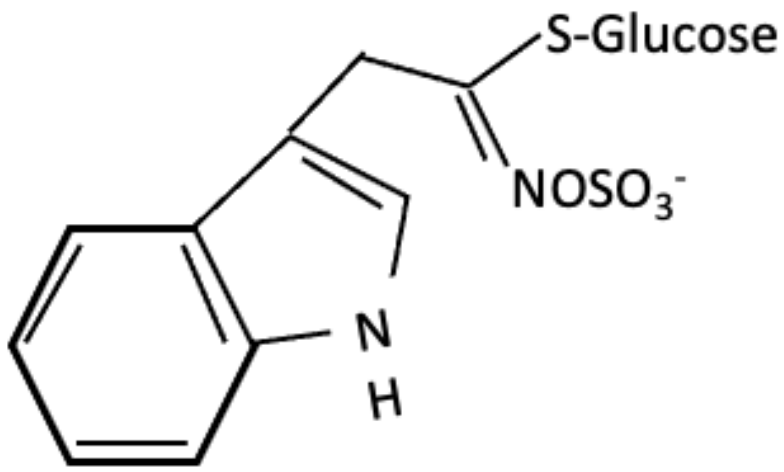

Figure 1.4. Indole-3-yl-methyl glucosinolate core structure. Adapted from Pfalz et al. (2009).

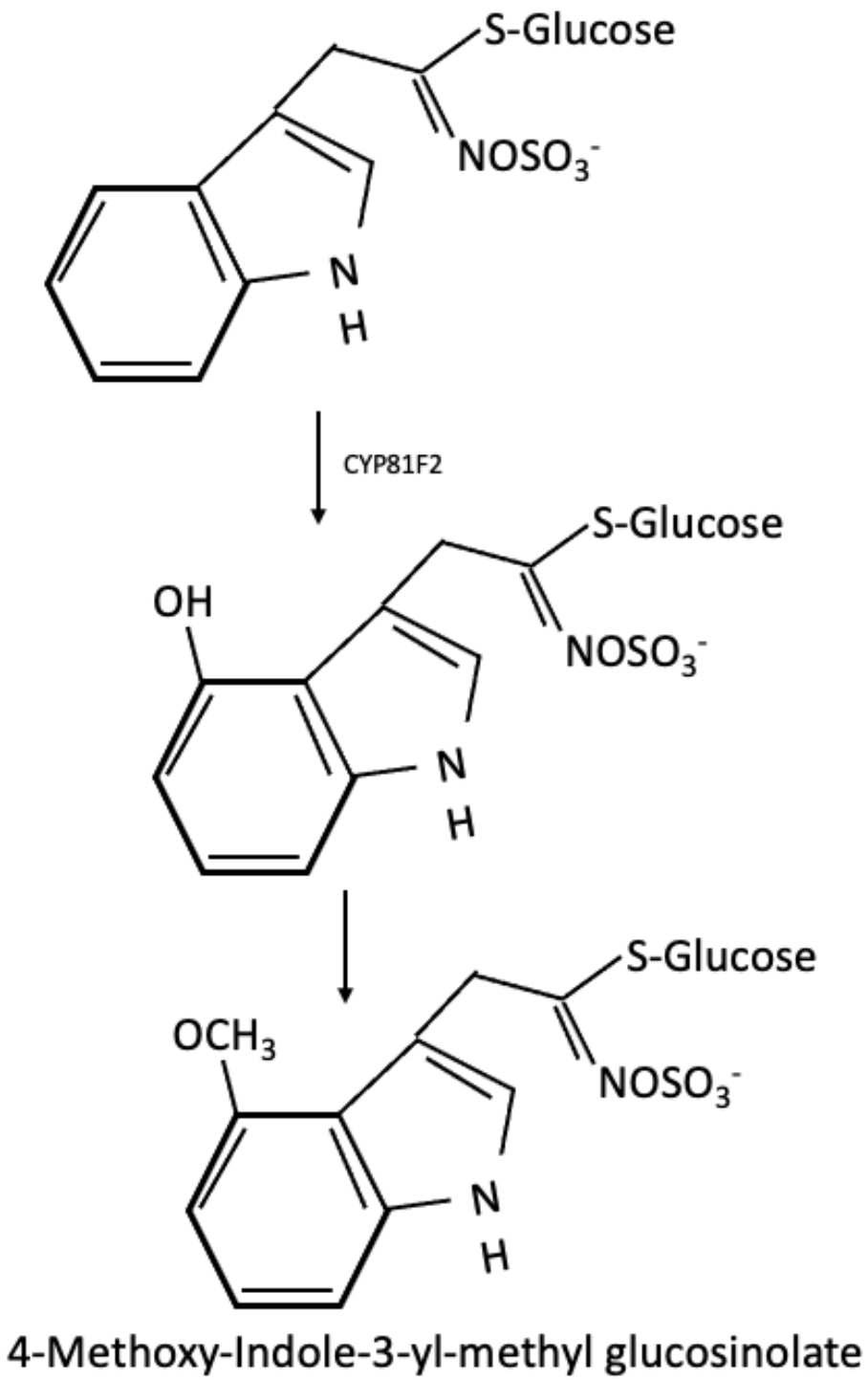

Figure 1.5. Secondary side chain modification pathway of Indole-3-yl-methylglucosinolate to 4-Methoxy-Indole-3-yl-methyl glucosinolate. Adapted from Pfalz et al. (2009)

activity of the degradation products (Hansen and Halkier 2005). The large number of GSLs suggests that specific hydrolytic products may be designed for specific situations and stages of development (Bones and Rossiter 1996).

1.4.3 Glucosinolate in Pathogen Defense

In the *Brassicaceae* family both aliphatic and indole GSLs are the most important GSLs, producing powerful antifungal compounds when hydrolyzed (Robin et al. 2017). Hydrolysis of GSLs resulting in the production of antifungal compounds is mediated by the myrosinase enzyme (Thioglucosidases) (Lüthy and Matile 1984). The precise cellular location of myrosinase has been debated. The 'mustard oil bomb' hypothesis was first proposed suggesting that GSLs are localized in the myrosin grains and myrosinase is in the cytoplasm (Lüthy and Matile 1984). This provided an explanation as to how myrosinase accessed GSLs upon cell damage. Further studies revealed that myrosinase is localized in the myrosin grains, while GSLs are present in non-myrosin cells (Rask et al. 2000). Currently the 'mustard oil bomb' hypothesis that is widely accepted is that tissue damage releases myrosinase from the myrosin grains in cellular compartments where GSLs are present (Rask et al. 2000). Products of GSL hydrolysis include isothiocyanates formed at pH > 7 and nitriles at pH < 4 (Halkier and Du 1997). In addition, thiocyanates are formed from intermediate GSLs such as 2-propenyl-, benzyl-, or 4-(methylthio)butylGSLs, goitrins are formed when side chains are β-hydroxylated, and epithionitriles when there is a terminal double bond in the side chain in the presence of epithiospecifier proteins and Fe^{2+} (Halkier and Du 1997).

Despite the realization that GSLs participate in plant defense responses, the mechanisms regulating their production remain elusive (Mithen and Magrath 1992; Robin et al. 2017). Past research into GSL levels in response to *L. maculans* infection has failed to establish strong correlations between GSL levels and plant resistance in *Brassica* species (Mithen and Magrath 1992). However, these

studies only measured total GSL content but not individual compounds (Mithen and Magrath 1992). Furthermore, these studies only focussed on *B. napus* and *L. maculans* (Mithen and Magrath 1992). More recent work showed an upregulation of aliphatic and indolic GSLs in *B. rapa* plants infected with *L. maculans* (Robin et al. 2017). This is significant because it points out that potential gene integration involved in the GSL production pathway for plant defense could differ from *B. rapa* to *B. napus* (Robin et al. 2017). An example of this integration is apparent in Surpass 400, a rapeseed cultivar that obtained blackleg resistance from *B. rapa* subsp. *Sylvestris* (Marcroft et al. 2012). Besides containing the qualitative resistance gene *Rlm3,* Surpass 400 was also found to contain other genes including *BLMR1* and *BLMR2* (Dandena et al. 2019). Recently, a candidate gene, suspected to be contained in the *BLMR2* region, *CYP81F2-A10* was identified as a homolog of *CYP81F2* in *Arabidopsis* (Zhang et al. 2021); this gene was also found to be associated with increased indole GSL accumulation in seedlings of cabbage cultivars with moderate blackleg resistance (Robin et al. 2017). The significance of this gene discovery within canola cultivars is related to the GSL production pathway and the specific products and uses. Within indole GSLs, the function of *CYP81F2* is to modify side chain molecules on indole-3yl-methyl-GSLs(I3G) to produce 4-methoxy-(I3G) (Abdel-Farid et al. 2010). Clay et al. (2009) found further evidence showing that this gene is involved in pathogen defense mechanisms, where 4-methoxy-I3G was required for the Flagellin22 (Flg22)-induced callose response. Plants with mutated *CYP81F2* genes showed a lack of callose response when Flg22 was applied (Clay et al. 2009). In the same study, it was found that treatment of Flg22 caused a reduction in I3G and the authors proposed a mechanism of activation of myrosinase by Flg22 (Clay et al. 2009). In addition, it was also proposed that the "perception of Flg22 triggers both the biosynthesis and subsequent hydrolysis of 4-methoxy-I3G" (Clay et al. 2009).

In *Arabidopsis*, *CYP81F2* has been identified to encode a P450 monooxygenase that is vital for pathogen-induced accumulation of 4-methyoxy-I3G (Bednarek et al. 2009). When tissue damage occurs, this particular GSL is activated by β-thioglucoside glucohydrolase (PEN2 myrosinase) and breakdown products are vital for plant anti-fungal defense (Bednarek et al. 2009). It has been documented that PEN2-driven GSL metabolic pathway results in products that are fundamentally different in response to fungal interaction compared to insect interactions (Bednarek et al. 2009). This study also found that upon infection, there was a reduction in the levels of 4-methoxy-I3G in plants that contained the *CYP81F2* gene (Bednarek et al. 2009). Based on these results, and in agreement to Clay et al. (2009), it was proposed that *CYP81F2* and PEN2 are integrated and operate in the same pathway (Bednarek et al. 2009). However, upregulation of CYP81F2 (*Bol026044*) in *B. oleracea* var. *capitata* (moderately blackleg resistance cultivars) upon *L. maculans* infection increased indole GSL levels in seedlings (Robin et al. 2017). This suggests that there may be a different function of *CYP81F2* in indole GSL production, separate from the biosynthesis with PEN2.

Based on these premises, the objectives of this thesis are to 1) measure the level of CYP81F2-A10 transcripts during infection 2) evaluate glucosinolate levels, specifically 4-methoxy-indol-3-ylmethyl, in the early, post-inoculation stages of developed near-isogenic lines (NILs) containing *BLMR2* compared to the wildtype Westar. I hypothesize that the gene, speculated to be contained within the *BLMR2* locus, CYP81F2-A10 is involved in the 4-methoxy-indol-3yl-methyl GSL production pathway and will be upregulated in response to pathogen infection. This upregulation will coincide with upregulation of 4-methoxy-indol-3yl-methyl GSL concentration. Higher concentrations of 4-methoxy-indol-3yl-methyl GSL will result in a broad-spectrum response to *L. maculans* infection.

2.0 Evaluation of glucosinolate levels with high performance liquid chromatography in near isogenic lines of *Brassica napus* L. containing BLMR2

2.1 Abstract

The transcript level of *CYP81F2-A10* and the amount of 4-methoxy-indole-3-yl-methyl-glucosinolates were measured in three *Brassica napus* L. near isogenic lines (NILs) (containing CYP81F2-A10_Surpass400) and wild type Westar (containing CYP81F2-A10_Westar) challenged with *Leptosphaeria maculans* (Desm.) Ces. Et de Not. Cotyledons were inoculated with *L. maculans* and samples were analysed over a period of 8 days post inoculation (DPI). CYP81F2-A10 was upregulated in all samples regardless of allele. The content of 4-methoxy-indole-3yl-methyl-glucosinolates was determined using extraction techniques and high-performance liquid chromatography. Most samples (NILs and Westar) displayed some level of induction in 4-methoxy-indole-3yl-methyl-glucosinolate in response to *L. maculans*, ranging in changes in concentration of 18 - 364%. However, relative to Westar, two NILs showed levels of induction that were significantly larger when compared to their respective water control and when compared to Westar inoculated. Microscopic studies showed that the spread of infection in the NILs was slower relative to Westar and began to show visible differences in infection rate at time points that correlated with the higher level of induction of 4-methoxy-indole-3yl-methyl-glucosinolate. This research work suggests that the CYP81F2-A10_Surpass400 locus is involved in this resistance mechanism.

2.2 Introduction

Brassica napus L., commonly referred to as canola, is an economically important crop grown by producers across Canada, accounting for approximately 25% of all farm crop receipts (Canola Council of Canada 2023d). Over all sectors in the Canadian economy, canola contributes $29.9 billion annually, and is responsible for over 200,000 jobs (Canadian Canola Growers Association 2021). With the goal to reduce overall greenhouse emissions and climate change, and improve agricultural sustainability, there is increased pressure on canola breeding to achieve the sustainability targets set by the Canola Council of Canada (Canola Council of Canada 2013; Canadian Canola Growers Association 2021). Tolerance to biotic stress is one of the major desirable traits that would benefit the canola industry (Manitoba Agriculture 2022).

Among the pathogens responsible for decreased yield in canola, the fungus *Leptosphaeria maculans* (Desm.) Ces. Et de Not has been a major concern for producers since the 1970s (Gugel and Petrie 1992; Canola Council of Canada 2023c). The initial infection of *L. maculans* usually occurs during the early stages of plant development; the pathogen can invade the host through stomata and wounds on cotyledons and true leaves (Marcroft 2019). These initial phases are followed by the formation of stem cankers at the base of the plant, causing a reduction in nutrient uptake and water supply and weakening the base of the stem (Fernando et al. 2007). Due to the severity of the disease and the ability of the pathogen to overwinter, there has been a great effort to establish effective and reliable forms of disease management to reduce yield loss (Gugel and Petrie 1992; Ash 2000; Canola Council of Canada 2020).

By the 1990s, cultivars of *B. napus* with high levels of genetic resistance were made commercially available in Canada (Canola Council of Canada 2023c). The genetic mechanism operating in these cultivars has been referred to as ‘Gene-for-Gene Theory’ (qualitative resistance) (Flor 1971;

Delourme et al. 2006; Canola Council of Canada 2023c). This type of interaction places extreme selection pressure on the pathogen and promotes rapid mutational shifts in the pathogen environment (Brun et al. 2010). It has been suggested that integrating genes involved in quantitative resistance could help reduce the selection pressure, providing partial resistance to pathogen isolates that are compatible with the qualitative resistance genes (gene-for-gene theory), allowing for long-term use of cultivars (Brun et al. 2010). Despite these efforts, the mechanisms of quantitative resistance are not fully understood, and research on the identification of specific genes involved in quantitative resistance has been limited (Long et al. 2011). One of the genes associated with providing quantitative adult plant resistance is *BLMR2*, a homolog of *Arabidopsis thaliana* (L.) Heynh gene *CYP81F2* (Zhang et al. 2021). This gene is involved in modification of glucosinolates (GSL), specifically in the secondary side chain modification of indole GSL to the 4-methoxy-indole-3-yl-methyl GSL (Abdel-Farid et al. 2010). This specific GSL, upon breakdown by the enzyme myrosinase, releases powerful antifungal compounds that contribute to plant defense (Zhang et al. 2021). Full length cDNA sequencing and comparison of the 53.37 kb region in near isogenic lines (NILs) and Westar revealed six nucleotide changes in this candidate gene (Zhang et al. 2021). Of these changes, only one results in an amino acid change at amino acid 57, changing aspartic acid to glutamic acid (Zhang et al. 2021) (Supplemental Figures 4.1 and 4.2).

Glucosinolates are well characterized plant secondary metabolites involved in defense mechanisms (Zang et al. 2009). Glucosinolates include many heterogenous compounds, and individual GSL makeup among plant species and cultivars can vary significantly depending on genetic and environmental diversity (Zang et al. 2009). There are three main core structures within the GSL family deriving from the specific amino acid: aliphatic GSLs derive from methionine, aromatic GSLs from phenylalanine/tyrosine, and indole GSLs from tryptophan (Halkier and Du

1997). These core structures then generate hundreds of different GSLs by further side chain modification through the activity of several enzymes (Wang et al. 2011). In the specific case of *CYP81F2*, this enzyme adds a hydroxide side chain to the indole-3yl-methyl GSL core structure. This is then subsequently converted to a methylthaizolo-OCH_3 (Abdel-Farid et al. 2010). Further diversification of GSL products occurs when there is tissue disruption, resulting in the physical contact between GSL and myrosinase. The activity of this enzyme cleaves a glucose group, thus converting GSLs into bioactive substances depending on pH, type of glucosinolate, and active group (Figure 2.1) (Zang et al. 2009). The breakdown of many aliphatic and indolic GSLs have displayed antifungal properties in plants (Bednarek et al. 2009). In particular, the GSL breakdown product isothiocyanate has been shown to have powerful antifungal properties, likely due to their reaction with thiols, amines and alcohols to produce dithiocarbamates, thiourea, or *O*-thiocarbamate derivatives (Plaszkó et al. 2021).

The amount of GSLs present in the tissue often correlates to plant resistance. *Brassica oleracea* L. seedlings displaying high levels of resistance to *L. maculans* contained high levels of the aliphatic GSLs glucoiberverin and glucoerucin, as well as indolic GSLs glucobrassicin, and 4-methoxy-indole-3yl-methyl-GSL (Robin et al. 2020) . This observation suggests that an increase in these GSLs compounds plays a role in the resistance to *L. maculans*, in particular 4-methoxy-indole-3yl-methyl-GSL was shown to display a vital role in the resistance to L. *maculans* in *B. oleracea* (Robin et al. 2020). Based on this premises, the objectives of this thesis are to 1) measure the level of CYP81F2-A10 transcripts in three NIL (containing CYP81F2-A10_Surpass400) and Westar (containing CYP81F2-A10_Westar) and 2) evaluate glucosinolate

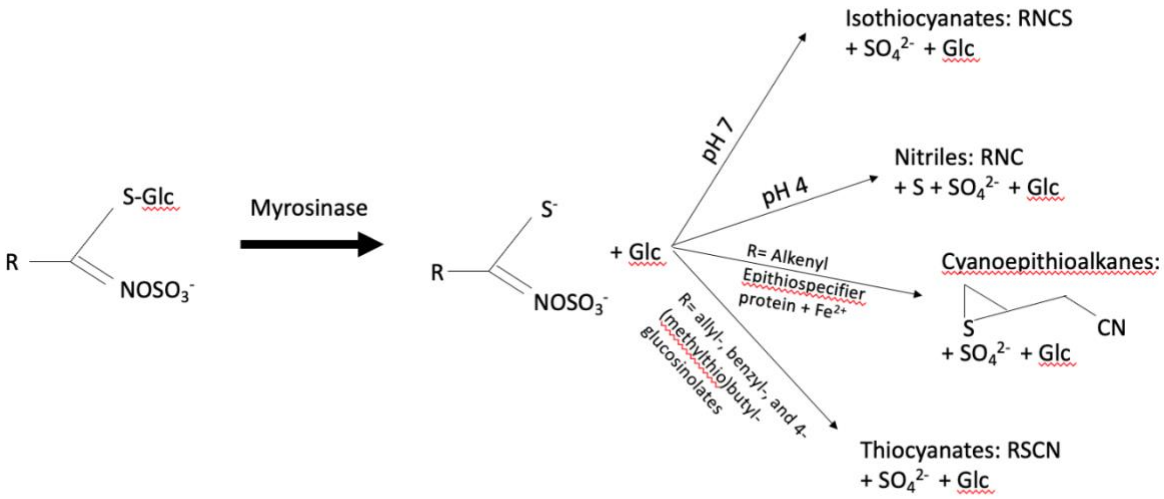

Figure 2.1. Degradation of glucosinolate by mirosinase and production of products based on the environment/structure. R= Core structure+ side chain. Adapted from Zang et al. (2009).

levels, specifically 4-methoxy-indol-3-ylmethyl, in the early stages of *L. maculans* infection in NILs and Westar.

2.3. Materials and Methods

2.3.1. Plant Materials

Three *B. napus* (NILs of Westar) containing *CYP81F2-A10_Surpass400* were previously developed via a series of crosses and backcrosses using cultivars Surpass 400 and Westar (Dandena et al. 2019; Zhang et al. 2021). Sequence information revealed that a single point mutation between alleles *CYP81F2-A10_Surpass400* and *CYP81F2-A10_Westar* resulted in an amino acid change at amino acid 57 from aspartic acid to glutamic acid (Supplemental material 4.1 and 4.2) (Zhang et al. 2021). For the current research, near isogenic lines Hn7, NJ11, and NJ13 were chosen to ensure a full coverage of the fine mapped 53.37 kb region of *CYP81F2-A10* identified by Zhang et al. (2021). All 3 lines were confirmed to have adult plant resistance (APR) to *L.* maculans strain 87-51 in Dandena et al. (2019).

2.3.2. Plant Growth Conditions

Wells of seed trays were pre-watered prior to planting with 5.6g/L 20-20-20 (nitrogen-phosphorous- potassium) fertilizer. Seed of each *B. napus* genotype was planted 1 cm deep in Sunshine® Mix #4 Aggregate Plus potting mix by Sungro Horticulture (Vancouver, BC). Seed trays were placed in controlled growth rooms with a photoperiod of 22 °C 16h light / 18 °C 8h dark, and a light intensity of 400 μmol s^{-1} m^{-2}. Seedlings were watered every other day as needed. Seedlings were grown for 10 days prior to inoculation, with true leaves being removed as they emerged to prevent cotyledon shedding.

2.3.3. *Leptospheria maculans* inoculation and sample collection

Ten days after planting, 50 % of the plants from each NIL and Westar were inoculated with *L. maculans* strain 87-41 (*AvrLm1* and *AvrLm2*) (Zhou et al. 2019). The other 50 % were treated in the same manner but, instead of a spore solution, distilled water was applied. Each cotyledon was lightly punctured with a pointed tweezer 4 times in the pattern shown in Figure 2.2 In each puncture, 10 µl of $2.5x10^7$ pycnidiospores per mL H_2O spore solution (or 10 µl of distilled water) was applied and the plants were incubated in the greenhouse at 19 °C for 16 h to ensure proper absorption before being placed back in the controlled growth room under the conditions listed above.

Plant cotyledon tissue was collected at 2, 4, 6, and 8 days post inoculation (DPI). Ten plants (20 cotyledons) were collected from each genotype for each treatment, with a total of 3 technical replicates. The cotyledons were immediately wrapped in tinfoil and placed in liquid nitrogen, they were then stored at -80 °C for GSL and RNA extraction.

2.3.4. Staining Technique

One cotyledon from each treatment and each NIL, plus Westar, was collected at 2, 4, 6, and 8 DPI and fixed in a solution of 60 % methanol, 30 % chloroform, and 10 % acetic acid (Bhadauria et al. 2010). Samples were rehydrated in decreasing concentrations of ethanol for 15 min each (100, 80, 70, and 50 %) before being stained with 0.05 % trypan blue in distilled water solution for 2 h (Bhadauria et al. 2010). Tissue was de-stained with distilled water and lightly shaken for 15 min three times (Bhadauria et al. 2010). Tissue was mounted on glass slides in 30 % glycerol and photographed. Images were visualized using a Laxco LMC3-BF4 Trinoc Microscope and a SeBaCam5C following manufacture protocol (Mill Creek, WA, USA). Software used was Laxco SeBaView version x64, 3.7.13725.20190106 (Mill Creek, WA, USA).

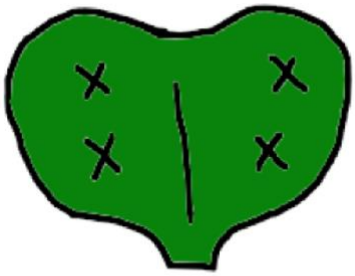

Figure 2.2 Drawing of cotyledon leaf with X to mark each puncture wound and solution application.

2.3.5. Glucosinolate Extraction

Extraction of GSLs was performed according to Kräling et al. (1990) and Li et al. (2001) with modifications. Samples were retrieved from -80 °C and placed in liquid nitrogen, 2 g of plant tissue were weighed and crushed using a mortar and pestle. Samples were placed in 15 ml polyethylene terephthalate (PET) centrifuge tubes and 7 ml of boiling 70 % methanol and 100 µl of sinigrin hydrate (0.07 mM) was immediately added. Tubes were shaken to ensure proper mixing and then placed in an 80 °C water bath for 10 min. Next, tubes were removed from the water bath and centrifuged for 15 min at 1900 g. In a separate centrifuge tube, 2 ml of diethylaminoethyl sephadex (DEAE-Sephadex) (1 g/ 15ml H_2O) was added and centrifuged for 5 min at 1900g. The supernatant was removed from the DEAE-Sephadex and 5 ml of the supernatant from the PET tubes was added. Tubes were briefly vortexed to ensure full incorporation of supernatant and DEAE-Sephadex, and incubated for 5 min at room temperature, before centrifuging for 10 min at 1900 g. After removing the supernatant, the pellet was washed with 70% methanol, centrifuged for 5 min at 1900 g and further washed twice with water. Finally, 20 µl sulfatase from *Helix pomatia* (Sigma-Aldrich 2022) (0.04g/ml) was added to the tubes and the samples were incubated at room temperature for 16 h. Three hundred µl H_2O was added and tubes were centrifuged for 10 min at 1900 g. The supernatant was stored at -20 °C before use for high-performance liquid chromatography.

2.3.6. HPLC Configuration

Forty ml of the GSL extract was run on a 5 mm column (Lichrocart 250–4 RP18e, EMD Chemicals, Darmstadt, Germany) on a Waters 2695 HPLC. Compounds were detected at 229 nm and separated by using aqueous acetonitrile. The program was an 8 min gradient from 1.5% to 5.0% (v/v) acetonitrile, a 2 min gradient from 5% to 7% (v/v) acetonitrile, a 32 min gradient from

7% to 52% (v/v) acetonitrile, a 2 min gradient from 52% to 92% (v/v) acetonitrile, 5 min at 92% (v/v) acetonitrile, a 3 min gradient from 92% to 1.5% (v/v) acetonitrile, and a final 8 min at 1.5% (v/v) acetonitrile (Kliebenstein et al. 2001).

2.3.7 Glucosinolate Calculation

Glucosinolate concentration was calculated using the known sinigrin concentrations and retention factors (Grosser and van Dam 2017). The internal control (sinigrin) concentration can be determined using the average area under the peak of 500000 and the known sample concentration (0.07 mM) (See Equation 1). The concentration of sinigrin is 0.19 mmol/g. Based on this result the concentration of 4-methoxy-indole-3yl-GSL was calculated using the known response factor (Table 2.1) and concentration of average sinigrin (See Equation 2)

(1)

$$x_t = \frac{x * M * D}{w}$$

x_t = Concentration of glucosinolates in the plant sample (μg/mol). x= amount of glucosinolates in the extract. D= Dilution factor. M= Response factor for detection at 229 nm. w= Mass of sample μg. (Grosser and van Dam 2017).

(2)

$$x_t = \left(\frac{x * 0.19}{500000}\right) * M$$

x_t = Concentration of glucosinolates in the plant sample (μg/mol). x= amount of glucosinolates in the extract. M= Response factor for detection at 229 nm. (Grosser and van Dam 2017).

Table 2.1. Response factor of various glucosinolates at 229 nm detection with HPLC (Buchner 1987; Brown et al. 2003; Grosser and van Dam 2017)

Glucosinolate	Response factor
Sinigrin	1
4-methoxyindol-3-ylmethyl	0.25

2.3.8 Internal Control

To identify the location of sinigrin by HPLC, 0.7 mM sinigrin solutions (100ul, 200ul and 300ul) were run, using the same protocol described for the GSL extraction in 2.3.5. The results show a peak at approximately 6 min with the area increasing with increasing volumes of sinigrin (Figure 2.2). Sinigrin Hydrate is a naturally occurring β-D-thioglucopyranoside that occurs in horseradish root and black mustard seeds, and is commonly used as a reference material for the identification and isolation of GSLs (Sigma-Aldrich 2021). Sinigrin hydrate is degraded by myrosinase in the same fashion that the GSLs of interest are degraded. Therefore, the level of sinigrin is used as an internal control to estimate myrosinase degradation of GSL in the experimental samples. The average area of sinigrin in all samples was 479320, with a standard deviation (σ) of 98008. Samples that were $\pm 2\sigma$ were eliminated. Using Univariate General Linear Model, the significance was determined to be 0.427, indicating no significant variation among levels of sinigrin.

2.3.9 Identification of 4-methoxy-indole-3yl-glucosinolate by HPLC

To determine the correct peak for 4-methoxy-indole-3ylmethyl-GSL, glucobrassicin potassium salt was used (Sigma-Aldrich 2022b). 0.7 mM glucobrassicin potassium salt solutions (100ul and 200ul) were run using the same protocol as described for the GSL extraction in 2.3.5. (Figure 2.4). By comparing our results with those of (Grosser and van Dam 2017), the retention time of 18.2 min for glucobrassicin, and the sequence of peak appearance for the other GSLs, suggested a retention time of 21.5 min for 4-methoxyindol-3-yl-methyl-GSL (Table 2.2 and Supplemental Figure 3.4). Therefore, this retention was used to quantify 4-methoxyindol-3-yl-methyl-GSL in the NILs and Westar.

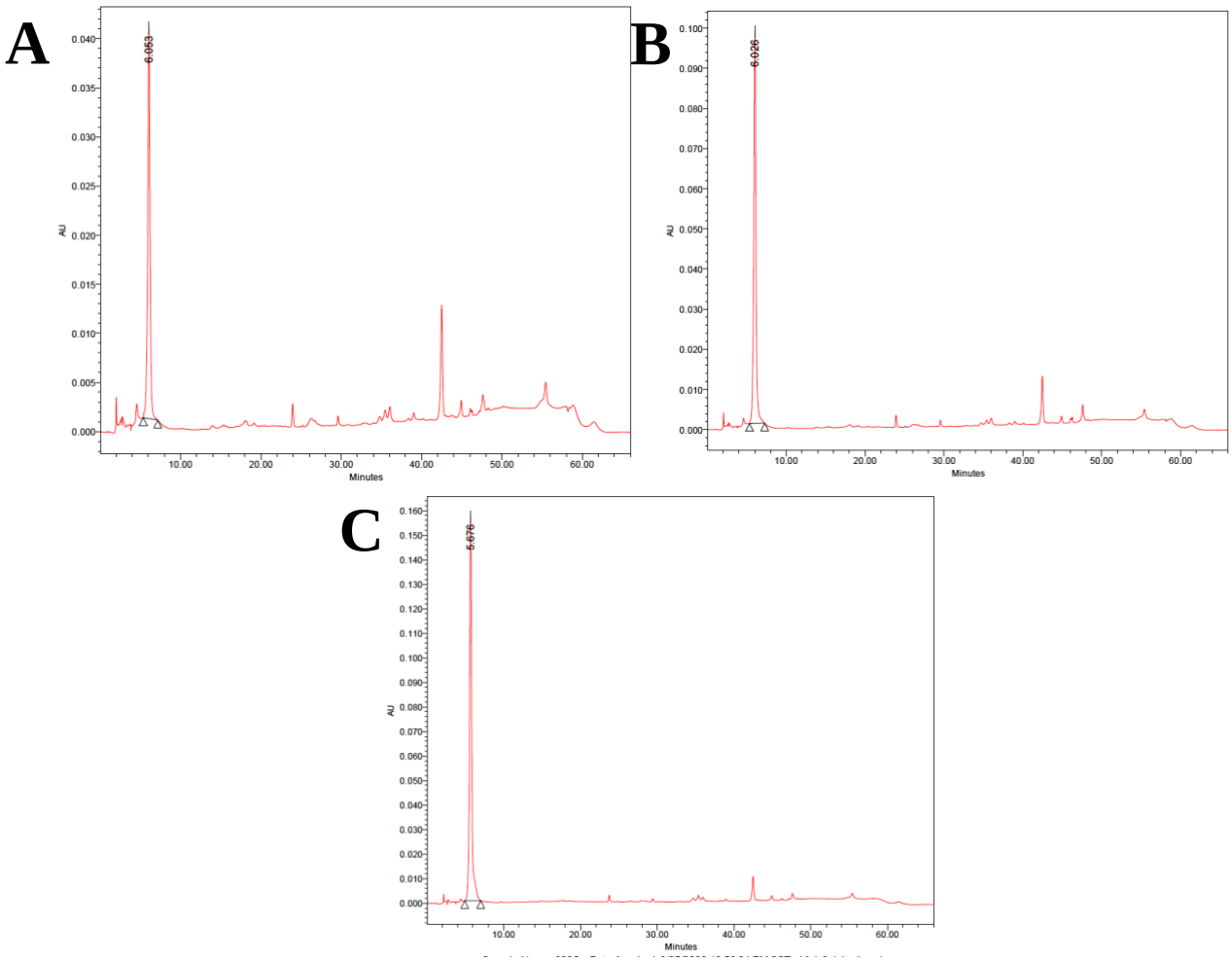

Figure 2.3. HPLC results for 100µl (A), 200 µl (B), and 300 µl (C) 0.7mM sinigrin sample. Peak with a retention time of 6.053 minutes (100 µl), 6.026 minutes (200 µl), and 5.676 minutes (300 µl) with an area of 796601 (100 µl), 1928892 (200 µl), and 3154061 (300 µl) were observed.

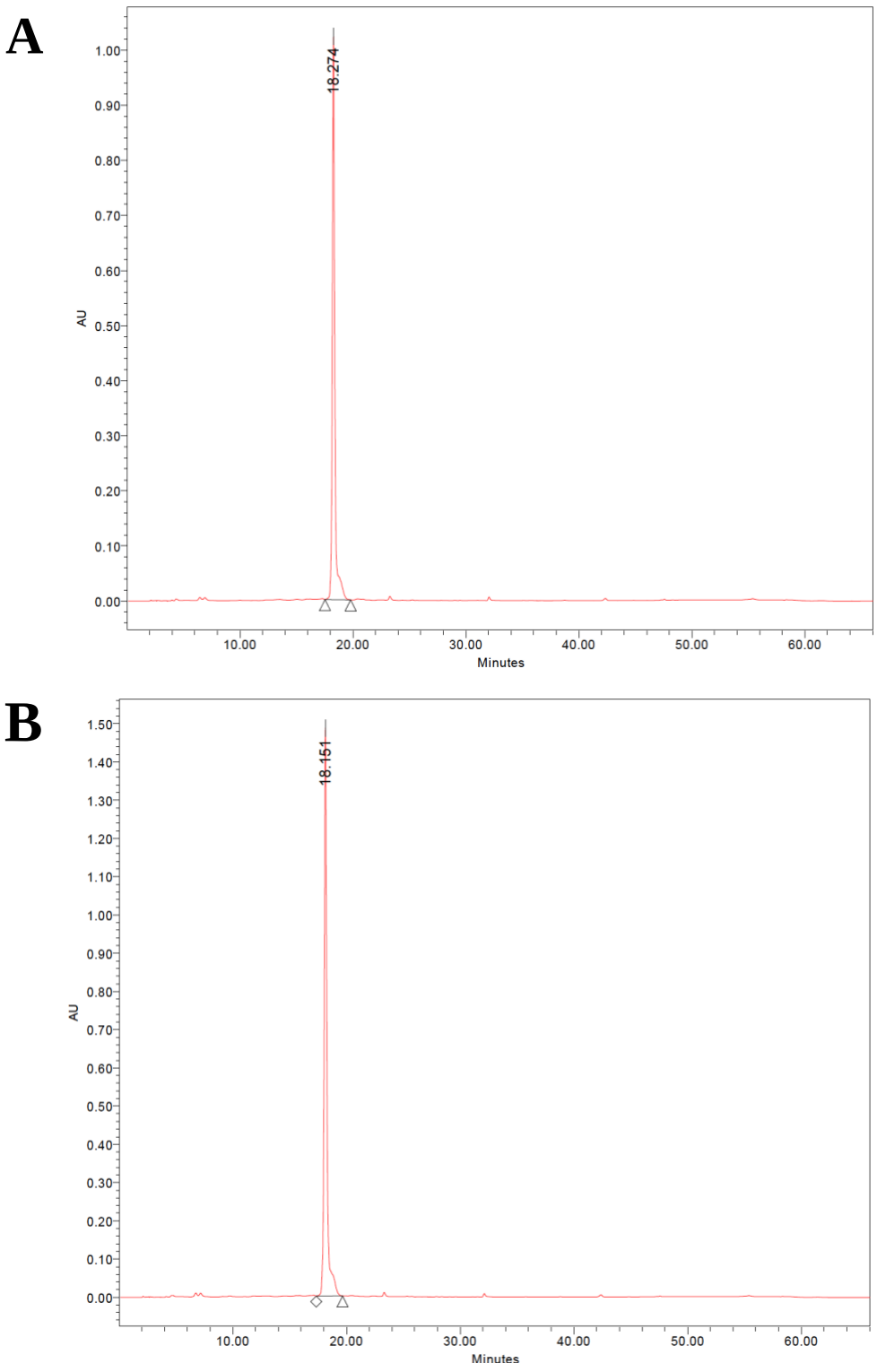

Figure 2.4. HPLC results for 100µl (A) and 200 µl (B) 0.7mM glucobrassicin sample. Peak with a retention time of 18.274 minutes (100 µl) and 18.151 minutes (200 µl) with an area of 15797039 (100 µl) and 25141563 (200 µl) were observed.

Table 2.2. Comparative glucosinolate retention times from glucobrassicin to 4-methoxyglucobrassicin reported by Grosser and van Dam (2017) with that reported in this study.

Common name	Side Chain Structure	Rt (min)[a]	Rt (min)[b]
Glucobrassicin	Indol-3-ylmethyl	15.3	18.2
Glucohirsutin	8-methylsulfinyloctyl	16.8	20.3
Glucoasturiin	2-phenylethyl	18.0	20.6
4-methoxyglucobrassicin	4-methoxyindol-3-ylmethyl	18.2	21.5

[a]From Grosser and van Dam 2017
[b]Retention times for the equipment and protocol utilized in this research.

2.3.8. RT-qPCR

Tissue for RT-qPCR was collected at the same time as tissue for GSL extraction. Two grams was used for GSL extraction, and the remainder was saved for RT-qPCR. RNeasy Plant Mini Kit (QIAGEN) was used to extract RNA, and High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, Massachusetts, United States) for cDNA synthesis. The protocol followed standard Quick-Start Protocol supplied with the kit following some minor modifications (Qiagen 2016). Just prior to extraction, 10 ml b-mercaptoethanol was added to 100 mg of frozen tissue. After the addition of 450 ml RLT buffer, the tissue was immediately ground and the lysate was transferred to a QIAshredder spin column with a 2 ml collection tube and centrifuged at 12000 g for 2 min. Supernatant was transferred to a new microcentrifuge tube and 200 ml 100 % ethanol was immediately added. Sample was transferred to a RNeasy Mini spin column with a 2 ml collection tube and centrifuged at 8000 g for 15 s and the flow through was discarded. Seven hundred ml RW1 buffer was added to the RNeasy spin column and centrifuged at 8000 g for 15 s, the flow through was discarded. Five hundred ml RPE Buffer was added to the RNeasy spin column and centrifuged at 8000 g for 15 s. Next, 500 ml RPE Buffer was added to the pellet in the RNeasy spin column and centrifuged at 8000 g for 2 min. Each spin column was added to a new 2 ml collection tube and centrifuged at 12000 g for 2 min to dry the membrane. The tube cap was opened for 3 min before the column was placed in a 1.5 ml collection tube and 30 ml RNase-free water was added directly to the spin column. The column was set for 5 min before being centrifuged at 8000 g for 1 min to elute the RNA. Concentration and quality of RNA were determined using a NanoDrop 2000 spectrophotometer (ThermoScientific, Waltham, Massachusetts, United States) following the manufactures protocol.

cDNA was generated using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's protocol. To each sample, 2 ml buffer, 0.8 ml dNTPs, 1 ml enzyme, 2 ml primer, and 4.2 ml DEPC water were added. Tubes were spun down and then incubated at 25 °C for 15 min, 37 °C for 2 hr, and then 85 °C for 5 min.

Quantitative real time PCR (qRT-PCR) was used to measure the relative gene expression of CYP81F2-A10 using the primer set MM1 amplifying both CYP81F2-A10_Surpass400 and CYP81F2-A10_Westar alleles. Reverse transcriptase products were diluted using a 3:1 ratio (water:cDNA), 4 µL was used for each reaction. The qRT-PCR was carried out using a 10 µL reaction volume using SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Hercules, California, USA). A C1000™ Thermal Cycler with CFX96™ Real Time System (Bio-Rad Laboratories, Hercules, California, USA) was used with reaction conditions described by (Elhiti et al. 2021). Relative Transcript levels were calculated and normalized using actin as an internal control. Fold-change values were calculated using the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

The primers used in gene expression study, MM1 from Dandena et al. (2019), amplified the CYP81F2-A10 on chromosome A10 and additional sequences on chromosomes A09, C09, C03, A03, and one unassigned 300-500 base pair region in the Westar genome.

2.3.9 Statistical Analysis

All statistical analysis was performed using IBM® SPSS® Statistics Software version 29.0.1.0 (IBM Canada Ltd., Markham, Ontario, Canada). In all statistical analysis, the P value of 0.05 was used for significance. Univariate General Linear Model was performed on the levels of sinigrin from the HPLC data, and on the 4-methoxy-indole-3yl-methyl-GSL levels in all samples inoculated with H_2O from HPLC data. In both tests, groups were separated by NIL. Independent-

sample t-tests were performed on RT-qPCR data to compare each NIL and Westar at 2, 4, 6, and 8 DPI. Independent-sample t-tests were performed on the HPLC data to compare each NIL and Westar inoculated with *L. maculans* strain 87-41 at 2, 4, 6, and 8 DPI. Independent-sample t-tests were performed on the HPLC data to compare each line's GSL level upon inoculation with *L. maculans* strain 87-41 versus inoculation with H_2O at the same time point.

2.4 Results

2.4.1. Phenotypic response of three NILs and Westar inoculated with *Leptospheria maculans*

The infection of *L. maculans* on the three NIL and Westar were assessed at 2, 4, 6, and 8 DPI. As no phenotypic differences were observed by 8 DPI (Figure 2.5), the assessment was extended to 14 DPI. At this timepoint, also selected by Dandena et al. (2019), the lesion size increased in Westar while it remained contained in the three NILs, especially in HN7. To better visualize the infection site, tissue was stained with trypan blue and observed microscopically (Figure 2.6). Formation of pycnidia around the inoculation site became apparent at 6 DPI in all samples. Development of pycnidia increased in all samples, except HN7, between 6 and 8 DPI, especially in Westar. This was in contrast to the NILs, displaying a moderate increase in pycnidia. Formation of pycnidia was reduced in the HN7 line from 6 to 8 DPI.

2.4.2. Expression of CYP81F2-A10 in response to inoculation with *Leptosphaeria maculans*

Expression of CYP81F2-A10 in the 3 NIL and Westar was examined at 2, 4, 6, and 8 DPI with *L. maculans* strain 87-41 (Zhang et al. 2021). Relative to the water control, inoculation with *L. maculans* increased the levels of CYP81F2-A10 transcripts in cotyledons of all lines (Table 2.3). The greatest induction was observed for HN7 at 6 DPI and this matched the low development pycnidia at 6 DPI to 8 DPI. When compared to Westar, all NILs at all time points were

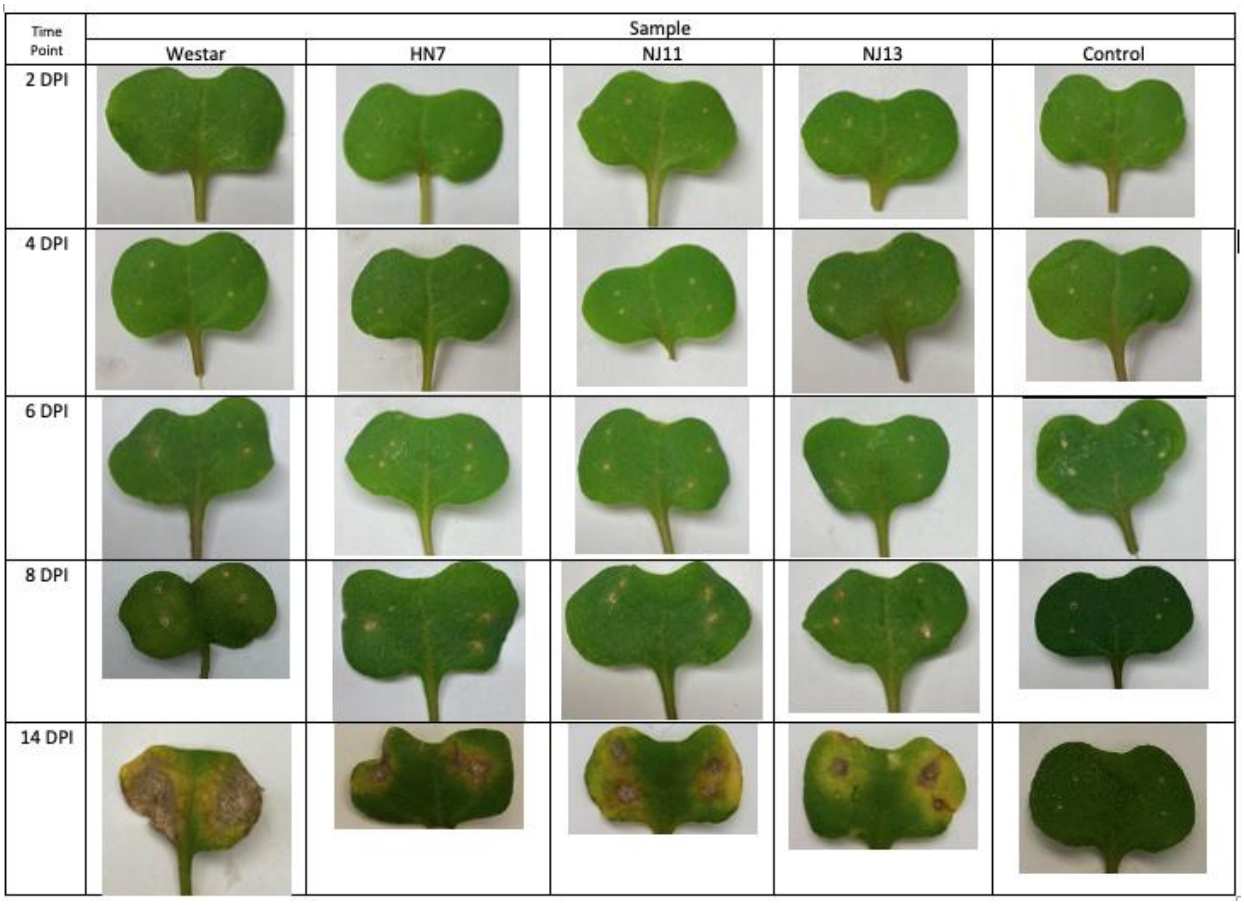

Figure 2.5. *Brassica napus* cotyledons inoculated with *Leptosphaeria maculans* strain 87-41. Cotyledons were inoculated 10 days after planting. A mock inoculation with water (control) was also included in Westar. Pictures taken at 2, 4, 6, 8, and 14 days post inoculation (DPI). Disease rating for 14 DPI is as follows Westar: 9, HN7: 3, NJ11: 3, NJ13: 2, Control: 0

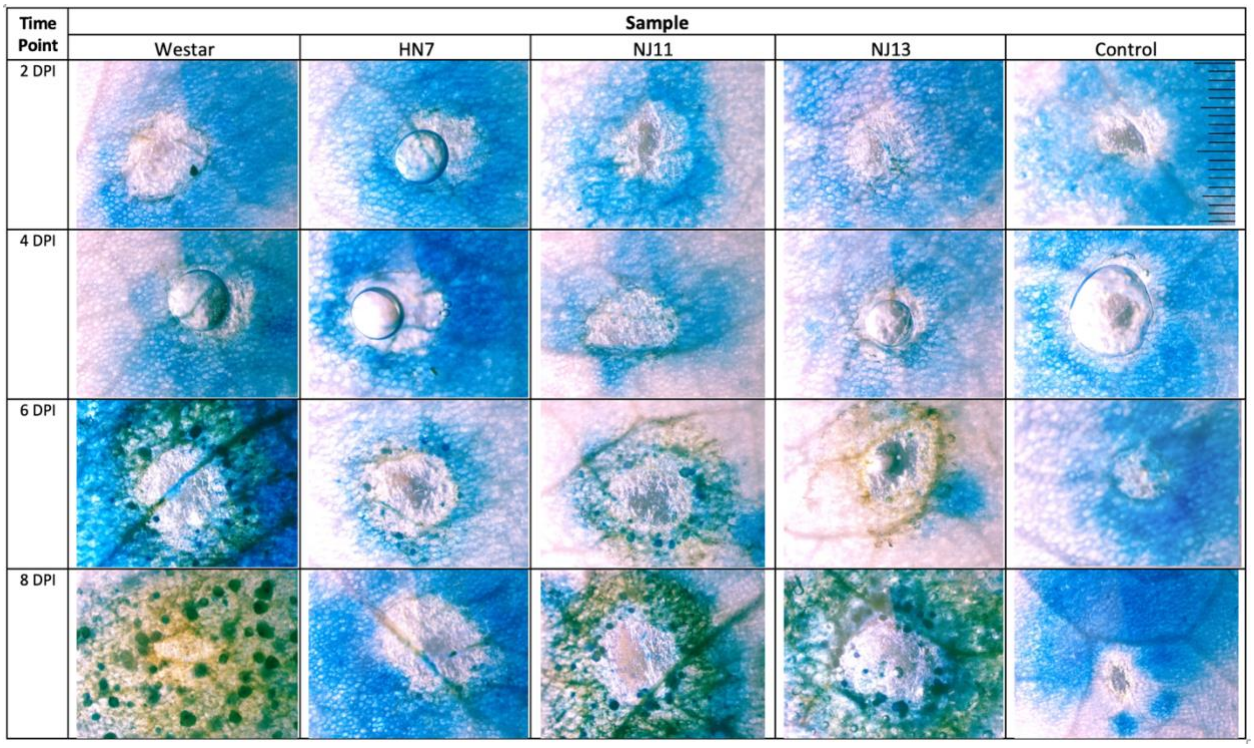

Figure 2.6. Histological examination of cotyledons inoculated with *Leptosphaeria maculans* strain 87-41 and stained with trypan blue. Plants were inoculated 10 days after planting and visualized at 2, 4, 6, and 8 days post inoculation (DPI). A mock inoculation with water (control) was also included in Westar. Scale bar = 0.1 μm

Table 2.3. RT-qPCR data depicting relative fold increase of *CYP81F2-A10* in inoculated samples of Westar and three NIL. Values are means ± SE of three biological replicates each consisting of 10 plants, and were normalized to the respective water control values set at 1. See supplementary table 4.1 for raw expression data.

Time Point	**Sample**			
	Westar(*CYP81F2-A10_Westar*)	HN7 (*CYP81F2-A10_Surpass400*)	NJ11 (*CYP81F2-A10_Surpass400*)	NJ13 (*CYP81F2-A10_Surpass400*)
2 DPI	1.40 ± 0.36	0.02 ±0.00	0.70 ±0.16	1.14 ±0.26
4DPI	5.56 ±0.79	2.84 ±0.45	2.54 ±0.34	0.88 ±0.08
6DPI	4.73 ±0.46	26.36 ±2.70	1.66 ±0.27	2.45 ±0.77
8DPI	8.38 ±0.47	2.22 ±0.42	12.33 ±1.43	2.64 ±0.50

significantly different, apart from NJ11 and NJ13 at 2DPI (Table 2.4) However, NJ13 did not behave in the same way as HN7 and NJ11, with fold increase remaining low at a maximum 2.64 compared to maximum of 26.36 in HN7 and 12.33 in NJ11. The expression in NJ13 was also lower vs Westar at 4, 6 and 8 DPI.

2.4.3.3. Quantification of 4-methoxyindol-3-ylmethyl-glucosinolate in three NILs and Westar inoculated with *Leptosphaeria maculans* strain 87-41

4-methoxyindol-3-yl-methyl-GSL was quantified in the three NIL and Westar following inoculation with *L. maculans* strain 87-41 at 2, 4, 6, and 8 DPI (Figure 2.7) and inoculation with H_2O (Figure 2.8). In all inoculated lines, the level of 4-methoxyindol-3-yl-methyl-GSL increased with time, reaching maximum values at 8DPI. The concentrations of 4-methoxyindole-3-yl-methyl-GSL were significantly higher in HN7 at 2 and 6 DPI and NJ11 at 6 DPI relative to Westar at the same timepoint when inoculated (Table 2.5). Univariate General Linear Model on 4-methoxy-indole-3yl-methyl-GSL levels of samples inoculated with H_2O showed a *P* value of 0.579, indicating no significant variance between genotypes (Figure 2.8). Apart from NJ11 at 2 DPI and Westar at 2 and 6DPI, inoculation increased the level of 4-methoxyindol-3-yl-methyl-GSL relative to the water control (Figure 2.7). When each inoculated line was compared to the same line and timepoint inoculated with H_2O, HN7 and NJ11 both show significant variation at 4 and 6 DPI and all lines showed statistically significant variation at 8 DPI (Figure 2.7).

Table 2.4. P-Values of Independent-Sample T Tests, on RT-qPCR data in inoculated samples of three NILs. RT-qPCR was normalized based on respective water control values set at 1. Each NIL compared to Westar at the same timepoint.

Time Point	HN7 (*CYP81F2-A10_Surpass400*)	NJ11 (*CYP81F2-A10_Surpass400*)	NJ13 (*CYP81F2-A10_Surpass400*)
2 DPI	0.003	0.052	0.285
4DPI	0.005	0.003	<0.001
6DPI	<0.001	<0.001	0.013
8DPI	<0.001	0.013	<0.001

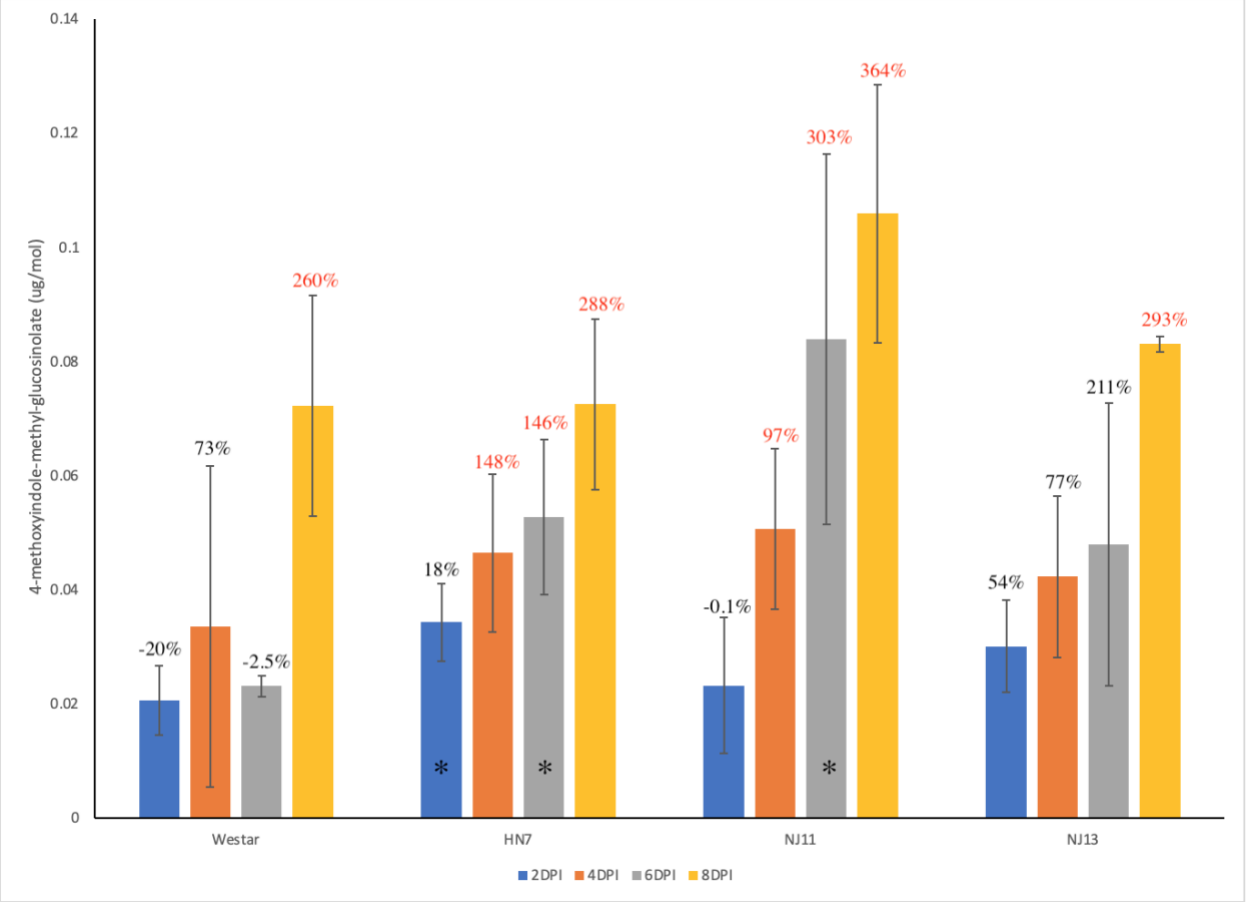

Figure 2.7. Total amount of 4-methoxyindole-3yl-methyl glucosinolate in three near isogenic lines and Westar at 2, 4, 6, and 8 days post inoculation (DPI) with *Leptosphaeria maculans* strain 87-41. Colored bars with error bars represent means ± standard error of three samples consisting of 10 plants for each genotype and time point, respectively. Percentages above the bars denote percent difference of 4-methoxyindole-3yl-methyl glucosinolate in inoculated vs control (H_2O) for each genotype at each timepoint. Percentages in red indicate inoculated values statistically significantly greater than their respective control. * Indicates time points that are statistically significantly higher than Westar at the same time points.

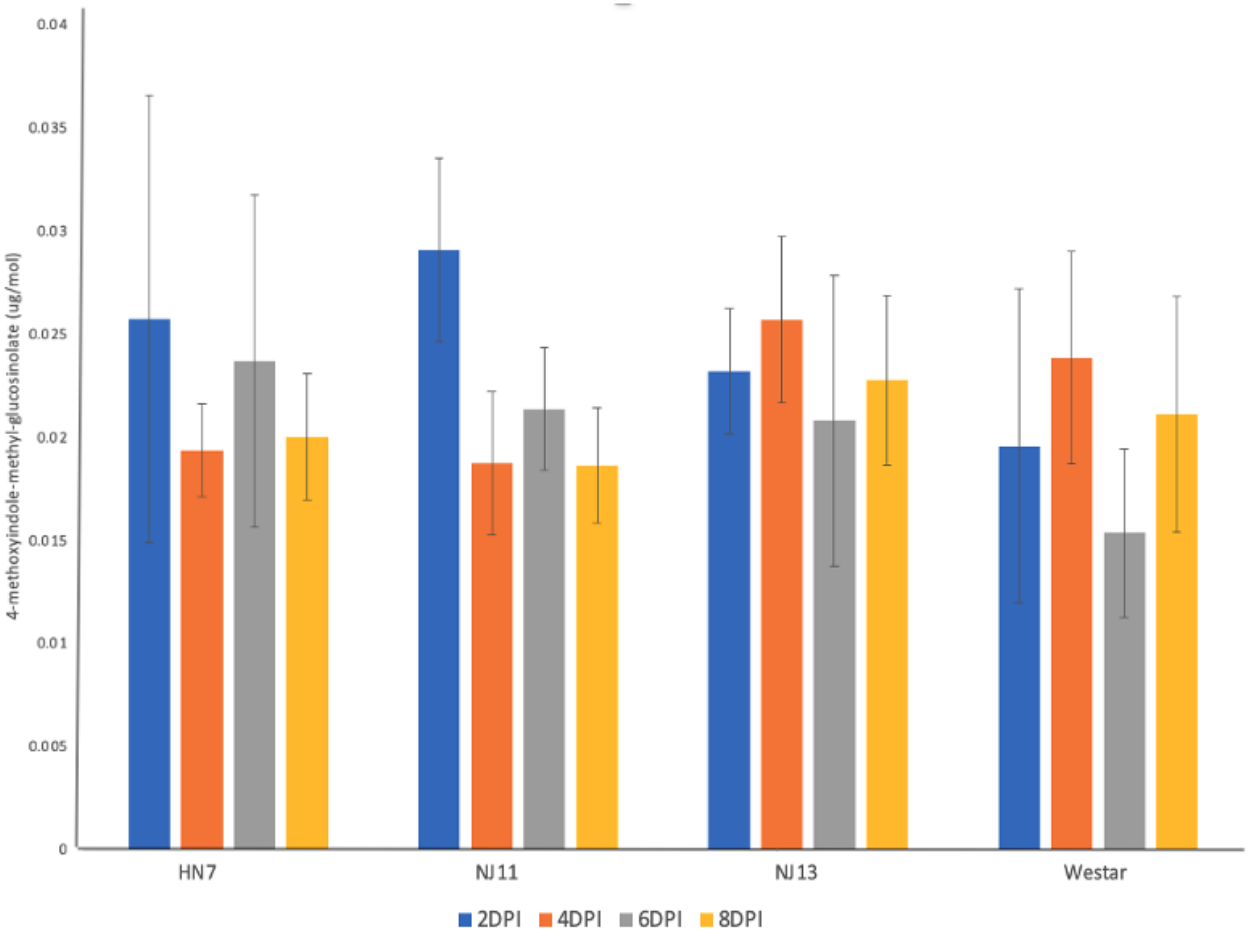

Figure 2.8. Total amount of 4-methoxyindole-3yl-methyl glucosinolate in three near isogenic lines and Westar at 2, 4, 6, and 8 days post inoculation (DPI) with water. Colored bars represent means of three samples consisting of 10 plants for each genotype and time point, respectively. Error bars represent ± standard error of three samples consisting of 10 plants for each genotype and time point, respectively.

2.5 Discussion

The evidence presented in this research suggests that the candidate gene *CYP81F2-A10,* found within the *Brassica napus* L. genome, is induced in response to inoculation with *L. maculans* and that the allele *CYP81F2-A10_Surpass400* could be involved in the conversion of secondary side chain modification of indolic GSL to 4-methoxyindol-3-yl-methyl-GSL. Previous studies have shown that *CYP81F2-A10_Surpass400* is present in the lines HN7, NJ11, and NJ13, but absent in Westar, which contains *CYP81F2-A10_Westar* (Zhang et al. 2021). The authors of the same study documented six polymorphisms within the candidate gene cDNA; however, only one mutation resulted in an amino acid change at position 57 (Supplemental material 3.1 and 3.2). It was further determined that *CYP8F2-A10* is homologous to the *Arabidopsis thaliana* gene *CYP81F2,* which functions in the modification of GSL secondary side chain from idole-3yl-methyl-GSL to 4-methoxy-indole-3yl-methy-GSL (Figure 1.5) (Zhang et al. 2021). In *Arabidopsis,* the expression of this gene, upon activation by the myrosinase PEN2 was shown to increase the accumulation of 4-methoxy-indole-3yl-methy-GSL (Bednarek et al. 2009). Studies on *Brassica oleraciea* L. cabbage cultivars with moderate blackleg resistance saw an upregulation of *CYP81F2* (Bol026044) in association with increased indole GSL accumulation at the seedling stage (Robin et al. 2017).

Glucosinolates are sulfur rich plant secondary metabolites derived from amino acids and sugars and have a large diversity based on secondary side chain modification (Halkier and Du 1997; Wang et al. 2011). Cellular disruption eliminates the barrier between myrosinase and GSLs resulting in various bioactive breakdown products (Sotelo et al. 2014). Many of these products greatly influence fungal and bacterial populations and are suspected to be major inhibitors of microbial activity (Sotelo et al. 2014). Specifically, the GSL breakdown product isothiocyanate has been

shown to be highly toxic and has significant inhibitory effects on fungal growth (Rahmanpour et al. 2009). Accumulation of GSLs depends on many different enzymes and genes (Giamoustaris and Mithen 1997). Not all GSLs have the same degree of reduction in pathogen growth, and increasing total GSLs has not been shown to provide a positive impact in controlling all pathogen populations (Giamoustaris and Mithen 1997). However, studies on both *B. oleracea* and *Brassica rapa* L. demonstrated that inoculation of resistant and susceptible plants with *Sclerotinia sclerotiorum* (Lib.) de Bary and *Mycosphaerella brassicicola* (Duby) Lindau, and *L. maculans* resulted in elevated aliphatic and indolic GSLs in resistant lines (Abdel-Farid et al. 2010; Robin et al. 2017; Abuyusuf et al. 2018). More specifically, studies have shown that, upon infection with fungal pathogens, there was an induction of 4-methoxy-indol-3yl-methyl-GSL. This indicates that this GSL, in the GSL-myrosinase system, plays a vital role in the plant defense mechanism (Bednarek et al. 2009). Further studies on the GSL accumulation in *B. oleracea* cultivars, both susceptible and resistant to *L. maculans,* upon infection showed that there was significant accumulation of pathogen induced GSL content at 2 and 4 DPI (Robin et al. 2020). These results were consistent with the results of previous studies on glucosinolates and demonstrated that an increase in either aliphatic GSL or indolic GSL was associated with seedling resistance (Robin et al. 2020). In particular, 4-methoxy-indol-3yl-methyl-GSL was shown to be significantly induced in resistant lines and decreased in susceptible lines (Robin et al. 2020).

When observing GSLs response to fungal inoculation and spread in *A. thaliana,* the enzyme *CYP81F2* was found to catalyze the accumulation of 4-methoxy-indole-3yl-methylGSL, whose hydrolysis products were directly transported to cells around fungal entry sites for antifungal defense (Bednarek et al. 2009; Chhajed et al. 2020). Additional studies found that that both PEN2 (myrosinase) and the transporter of 4-methoxy-indole-3yl-methyl-GSLs hydrolysis products are

required for microbe-associated molecular pattern triggered defense response (Clay et al. 2009; Chhajed et al. 2020). The findings of both of these studies suggests that hydrolysis of glucosinolate products plays a role in signaling molecules associated with plant immune response (Chhajed et al. 2020).

In some cases, *A.thaliana* has been shown to increase synthesis of 4 methoxy-indole-3yl-methyl-GSLs in its leaves in response to some insect damage, (*Myzus persicae* (Sulzer))(Kim and Jander 2007; Chhajed et al. 2020). However other insects do not cause the same response to GSL levels and have developed ways in which to evade inducing initiating signalling of increased production. For example, *Helicoverpa zea* (Boddie) feed on inner lamina of leaves due to GSLs tending to allocate mostly in the outer portion. This reduces the amount of damaged GSLs, therefore reducing signalling to increase GSL production (Chhajed et al. 2020). When analyzing insect resistance, research suggests that generalist resistance was higher in plants with higher glucosinolate content but specialist resistance was higher in plants with higher myrosinase activity (Hopkins et al. 2009). This is likely due to the fact that generalists are not able to process the breakdown products of glucosinolates once consumed, relative to specialists (Hopkins et al. 2009).

Genetic resistance to blackleg in *B. napus* is classified by two types of resistance, qualitative resistance and quantitative resistance (Flor 1971; Sprague et al. 2018; Cornelsen et al. 2021). Qualitative resistance is associated with R gene (gene-for-gene theory) resistance and is often associated with severe restriction of hyphal growth in cotyledons and true leaves (Delourme et al. 2006). Quantitative resistance is more often associated with reduced stem canker severity via limiting pathogen growth and development, mediated by polygenes (Hammond and Lewis 1987; Li et al. 2004; Delourme et al. 2008; Dandena et al. 2019). Polygenes are often associated with partial resistance which was seen in field trials of the NILs in this study, where they displayed an

intermediate disease severity index (Dandena et al. 2019). There is evidence that seedling and adult plant resistance can be correlated in some cases (Hammond and Lewis 1987). *BLMR2* has been hypothesized to be a gene involved in quantitative genetic resistance with partial seedling resistance that correlated to the intermediate adult plant resistance found in field trials (Dandena et al. 2019)

Due to the high similarity in nucleotide sequence between *CYP81F2-A10_Surpass400* and *CYP81F2-A10_Westar* (Zhang et al. 2021), the primers designed for qRT-PCR analyses were not able to distinguish between the two alleles. Our data show that the expression of *CYP81F2-A10,* upon inoculation with *L. maculans,* increased in all lines, suggesting that this gene could be involved in the plants response to infection. The increase in expression of this gene coincided with an increase in production of 4-methoxy-indol-3yl-methyl-GSL up to 6 DPI in all NILs but not in Westar (Table 2.3, Figure 2.7). These results suggest that *CYP81F2-A10_Surpass400* is a homolog of the *CYP81F2* gene and thus, allows for the additional conversion to 4-methoxy-indol-3yl-methyl-GSL. Whereas Westar, which contains *CYP81F2-A10_Westar,* did not see the same initial upregulation and associated resistance. Staining and microscopic imaging of inoculated cotyledons showed a reduction in pathogen growth and development in NILs compared to Westar beginning at 6 DPI (Figure 2.6). Analysis of water samples indicates that, in the absence of inoculum, there was no upregulation of GSL and that the NILs contained relatively low GSL initially, similar to Westar (Supplemental Table 3.5).

The upregulation in GSL at 8 DPI in Westar did not show any reduction or suppression of infection microscopically (Figure 2.4). This would suggest that multiple genes and pathways are involved in the increase in GSL content post 8 DPI and that early upregulation of 4-methoxy-indol3yl-methyl-GSL is essential in plant defense. Once infection was established, this GSLs breakdown

products were not significant enough in their antifungal properties to cause a visible limitation on the pathogen's growth and development.

These results correlate with those found by Dandena et al. (2019) in that the NILs shown to display intermediate adult plant resistance have also demonstrated partial seedling resistance. The study conducted by Zhang et al. (2021) together with the data presented here suggests that this specific allele, *CYP81F2-A10_Surpass400*, could potentially be the allele responsible for the *BLMR2* resistance locus.

3.0 Conclusion

Upon damage, plants from the *Brassicales* order hydrolyze glucosinolates to produce many antifungal products (Bednarek et al. 2009). The type and amount of products depend on the environmental conditions, loci, and the structure/side chain of the initial glucosinolate (GSL) (Halkier and Du 1997). Among the hundreds of GSLs, one of the most important GSLs in fungal defense is 4-methoxy-indol-3yl-methyl-GSL, whose hydraulic products have been shown to play a major factor in various resistance to pathogens (Pfalz et al. 2009; Chhajed et al. 2020; Zhang et al. 2021). However, GSLs in *Brassica napus* L. are undesirable in large quantities and breeding efforts focus on obtaining cultivars with low levels of total GSLs (Alexander et al. 2008). This thesis investigates the involvement of the candidate gene *CYP81F2-A10* in *B. napus*, a homolog of *CYP81F2* in *Arabidopsis thaliana* L., in the resistance response when inoculated with *Leptosphaeria maculans* (Desm.) Ces. Et de Not. In this study, all three NILs and Westar showed upregulation of the *CYP81F2-A10* allele upon inoculation with *L. maculans* in analysis with RT-qPCR. However, the fold increase in expression differed across genotypes and timepoints, and the maximum fold increase varied between 6 and 8 DPI. Upon inoculation with *L. maculans*, the NILs began to show phenotypic differences from the wild type (Westar) at 6DPI; this coincided with significant differences in the concentration of 4-methoxy-indol-3yl-methyl-GSL at the same time point. However, only two of the NILs containing *BLMR2*, the homolog of *CYP81F2*, showed a significant upregulation in 4-methoxy-indol-3yl-methyl-GSL at 6DPI and showed partial resistance to the pathogen. This evidence suggests that the *CYP81F2-A10_Surpass400* allele could be responsible for the resistance seen in the NILs.

3.1 Future direction

The NILs used in this study were developed via a series of crosses between Surpass 400 and Westar to integrate the *BLMR2* locus into the NILs. To ensure the segment in question is responsible for the increase in production of 4-methoxy-indol-3yl-methyl GSL the experiment should be repeated using transgenic plants in which the gene is knocked out or complemented via CRISPR-Cas9 or a transformation protocol. This information would provide stronger evidence in confirming the role of this gene in quantitative resistance to *L. maculans*. Additionally, the primers used in RT-qPCR did not distinguish between those with and those without the point mutation causing the amino acid change, to specify further it would require designing KASP primers to distinguish those with the single point mutation and those without. The results demonstrated that there was upregulation in all lines, regardless of the allele. It would be beneficial to be able to distinguish between those with or without the mutation to provide further evidence that this specific upregulation is the cause of the upregulation of GSLs seen, and not some other upregulation happening coincidently.

It would also be beneficial to test the ability of this mechanism to provide added protection when there is pathogen mutational shift. As quantitative resistance to *L. maculans* has not been shown to provide complete resistance, it is suggested to be introgress into lines that contain qualitative resistance mechanisms. Therefore, it would be beneficial to test the effectiveness of this resistance to provide added protection and preserve qualitative resistance mechanisms against other races of the pathogen. If this gene provides resistance to multiple races of the pathogen, it could be extremely useful to commercial canola breeding programs.

Supplementary Material

```
Westar          ATGGATTACATTTTGCTCTTATTGCCACTCGTATTGTTTCTACTAGCTTACAAATTCTTA
W+BLMR2         ATGGATTACATTTTGCTCTTATTGCCACTCGTATTGTTTCTACTAGCTTACAAATTCTTA
                ************************************************************

Westar          TTCTCATCTAAGAGTTTCAATCTTCCACCAGGACCAACTCCCTTTCCCATCGTCGGCAAC
W+BLMR2         TTCTCATCTAAGAGTTTCAATCTTCCACCAGGACCAACTCCCTTTCCCATCGTCGGCAAC
                ************************************************************

Westar          CTCCACCTCGTGAAACCACCGGTGCACCGTCTCTTCCGTCGTTTCGCGGACAAGTACGGT
W+BLMR2         CTCCACCTCGTGAAACCACCGGTGCACCGTCTCTTCCGTCGTTTCGCGGAGAAGTACGGT
                ************************************************** *********

Westar          GACATCTTCTCCCTCCGTTACGGCTCTCGCCAAGTCGTCGTGATCTCTTCCTTGCCCCTC
W+BLMR2         GACATCTTCTCCCTCCGTTACGGCTCTCGCCAAGTCGTCGTGATCTCTTCCTTGCCCCTC
                ************************************************************

Westar          GTCAGAGAATGCTTTACTGGTCAGAACGACGTTATTTTAACGAACCGACCGCATTTTCTG
W+BLMR2         GTCAGAGAATGCTTTACTGGTCAGAACGACGTTATTTTAACGAACCGACCGCATTTTCTG
                ************************************************************

Westar          ACCGCAAAGTACGTTGCTTACGACTACACCACGGTTGGAACCGCCGCATATGGCGACCAC
W+BLMR2         ACCGCAAAGTACGTTGCTTACGACTACACCACGGTTGGAACCGCCGCATATGGCGACCAC
                ************************************************************

Westar          TGGCGTAATCTCCGCCGTATTTGCTCTCTTGAGATCCTTTCCTCTAACCGTCTCACTGGA
W+BLMR2         TGGCGTAATCTCCGCCGTATTTGCTCTCTTGAGATCCTTTCCTCTAACCGTCTCACTGGA
                ************************************************************

Westar          TTCCTCTCCGTTCGTAAAGACGAGATCCGACGGTTGCTCACGAAACTCTCACGTGACTAT
W+BLMR2         TTCCTCTCCGTTCGTAAAGACGAGATCCGACGGTTGCTCACGAAACTCTCACGTGACTAT
                ************************************************************

Westar          AATGGCCAAGTCGTTGAGCTTGAGCCTCTTCTTGCAGATTTGACGTTCAATAATATTGTC
W+BLMR2         AATGGCCAAGTCGTTGAGCTTGAGCCTCTTCTTGCAGATTTGACGTTCAATAATATTGTC
                ************************************************************

Westar          CGTATGGTCACTGGGAGACGTTACTACGGAGACCAGGTTCACAACAAGGAAGAAGCGAAC
W+BLMR2         CGTATGGTCACTGGGAGACGTTACTACGGAGACCAGGTTCACAACAAGGAAGAAGCGAAC
                ************************************************************

Westar          CTATTCAAGAAGCTAGTGACGCAGATCAACGACAATAGTGGTGCGAGCCATCCAGGAGAT
W+BLMR2         CTATTCAAGAAGCTAGTGACGCAGATCAACGACAATAGTGGTGCGAGCCATCCAGGAGAT
                ************************************************************

Westar          TATTTACCAATTCTCAAAGTTTTCGGACACGGCTACGAGAAGAAAGTGAAAGCACTCGGC
W+BLMR2         TATTTACCAATTCTCAAAGTTTTCGGACACGGCTACGAGAAGAAAGTGAAAGCACTCGGC
                ************************************************************

Westar          GAAGCCATGGACACTTTCTTGCAGCGACTGCTCGACGATTGCCGTAGAGATGGAGAGAGC
W+BLMR2         GAAGCCATGGACACTTTCTTGCAGCGACTGCTCGACGATTGCCGTAGAGATGGAGAGAGC
                ************************************************************

Westar          AACACAATGCTTAGTCATCTGTTGTCTTTACAAGTAGACCAACCCAAGTATTACAGTGAC
W+BLMR2         AACACAATGCTTAGTCATCTGTTGTCTTTACAAGTAGACCAACCCAAGTATTACAGTGAC
                ************************************************************

Westar          GTCATCATCAAAGGCCTCATGCTCAGTATGATGCTTGCGGGGACGGATACTGCAGCCGTG
W+BLMR2         GTCATCATCAAAGGCCTCATGCTCAGTATGATGCTTGCGGGGACGGATACTGCAGCCGTG
                ************************************************************
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Westar          ACACTAGAATGGGCGATGGCGAGTTTGTTGAAAAGTCCTGAAGTGTTGAAGAAGGCGAAA
W+BLMR2         ACACTAGAATGGGCGATGGCGAGTTTGTTGAAAAGTCCTGAAGTGTTGAAGAAGGCGAAA
                ************************************************************

Westar          GCCGAGATAGATGATAAGATTGGACATGAACGTTTGGTCGACGAACCGGACATTTTGAAT
W+BLMR2         GCCGAGATAGATGATAAGATTGGACATGAACGTTTGGTCGACGAACCAGACATTTTGAAT
                ************************************************.***********

Westar          CTCCCTTATCTCCAAAACATAGTTTCTGAGACCTTCCGACTGTGTCCAGCCGCACCACTC
W+BLMR2         CTCCCTTATCTCCAAAACATAGTTTCCGAGACCTTCCGACTGTGTCCAGCCGCACCACTC
                ************************** ***********************************

Westar          CTTGTACCACGTTCTCCTTCTGAAGACCTCAAGATTGGCGGATACGACATACCGCGTGGC
W+BLMR2         CTTGTCCCACGTTCTCCTTCTGAAGACCTCAAGATTGGCGGATACGACATACCGCGTGGC
                *****.******************************************************

Westar          ACCATCGTACTAGTGAATTCTTGGGCCATCCATAGAGATCCAAGGCTTTGGGATGAGCCT
W+BLMR2         ACCATCGTACTAGTGAATTCTTGGGCCATCCATAGAGATCCAAGGCTTTGGGATGAGCCT
                ************************************************************

Westar          GAGAGGTTCATGCCAGAGCGGTTTGAGGACAAAGAAGCTGCCAATAATAATAAGCTTATG
W+BLMR2         GAGAGGTTCATGCCAGAGCGGTTTGAGGACAAAGAAGCTGCCAATAATAATAAGCTTATG
                ************************************************************

Westar          ATGTTTGGGAACGGACGAAGGACGTGTCCCGGTGCGGCTTTGGGTCAAAGGATGGTGTCG
W+BLMR2         ATGTTTGGGAACGGACGAAGGACGTGTCCCGGTGCGGCTTTGGGTCAGAGGATGGTGTCG
                ************************************************.***********

Westar          TTGGCTTTAGGATCGTTGATTCAATGCTTTGACTGGGAAAAAGTCAACGGTGAGGAAATT
W+BLMR2         TTGGCTTTAGGATCGTTGATTCAATGCTTTGACTGGGAAAAAGTCAACGGTGAGGAAATT
                ************************************************************

Westar          GATATGACCGAAAATCCTGGAATGGCTATGCGCAAGCTCGTGCCGTTACGAGCCGTTTGC
W+BLMR2         GATATGACCGAAAATCCTGGAATGGCTATGCGTAAGCTCGTGCCGTTACGAGCCGTTTGC
                ********************************* ****************************

Westar          CATCAGCGTCCCATTATGACTAATCTTTTGGCTTAA
W+BLMR2         CATCAGCGTCCCATTATGACTAATCTTTTGGCTTAA
                ************************************
```

Supplemental Figure 4.1. Comparison in cDNA sequence of the CYP81F2 homolog between Westar and the developed lines containing *BLMR*2 (W+*BLMR*2). Zhang et al. (2021).

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Westar          MDYILLLLPLVLFLLAYKFLFSSKSFNLPPGPTPFPIVGNLHLVKPPVHRLFRRFADKYG
W+BLMR2         MDYILLLLPLVLFLLAYKFLFSSKSFNLPPGPTPFPIVGNLHLVKPPVHRLFRRFAEKYG
                ************************************************************:***

Westar          DIFSLRYGSRQVVVISSLPLVRECFTGQNDVILTNRPHFLTAKYVAYDYTTVGTAAYGDH
W+BLMR2         DIFSLRYGSRQVVVISSLPLVRECFTGQNDVILTNRPHFLTAKYVAYDYTTVGTAAYGDH
                ************************************************************

Westar          WRNLRRICSLEILSSNRLTGFLSVRKDEIRRLLTKLSRDYNGQVVELEPLLADLTFNNIV
W+BLMR2         WRNLRRICSLEILSSNRLTGFLSVRKDEIRRLLTKLSRDYNGQVVELEPLLADLTFNNIV
                ************************************************************

 Westar          RMVTGRRYYGDQVHNKEEANLFKKLVTQINDNSGASHPGDYLPILKVFGHGYEKKVKALG
 W+BLMR2         RMVTGRRYYGDQVHNKEEANLFKKLVTQINDNSGASHPGDYLPILKVFGHGYEKKVKALG
                 ************************************************************

 Westar          EAMDTFLQRLLDDCRRDGESNTMLSHLLSLQVDQPKYYSDVIIKGLMLSMMLAGTDTAAV
 W+BLMR2         EAMDTFLQRLLDDCRRDGESNTMLSHLLSLQVDQPKYYSDVIIKGLMLSMMLAGTDTAAV
                 ************************************************************

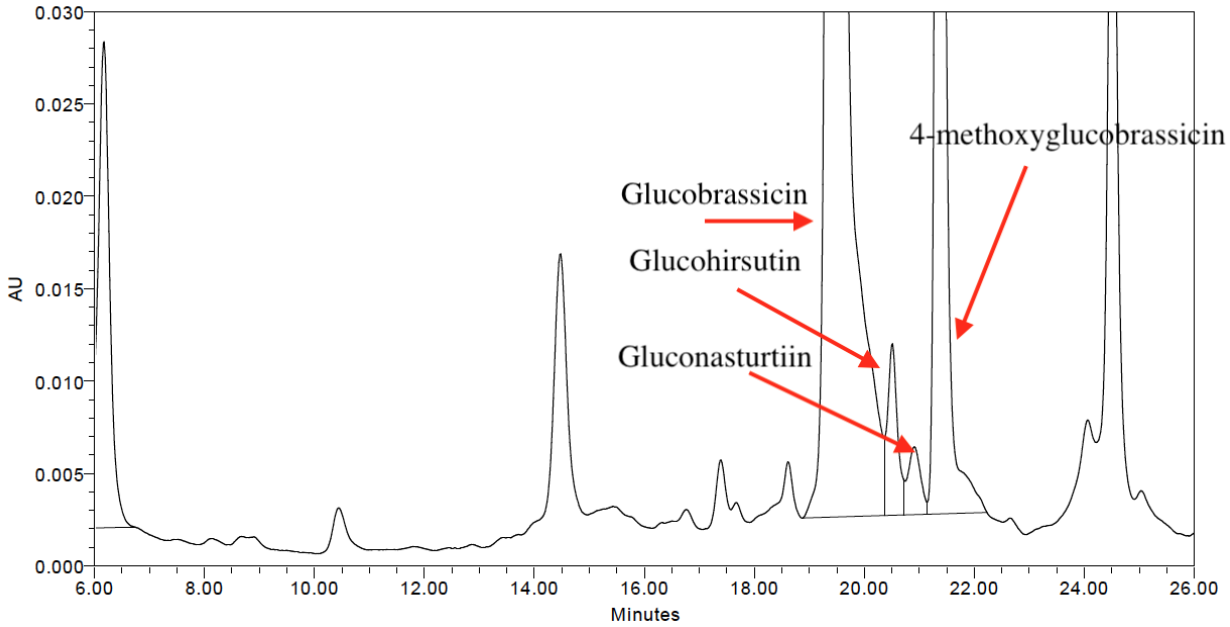
 Westar          TLEWAMASLLKSPEVLKKAKAEIDDKIGHERLVDEPDILNLPYLQNIVSETFRLCPAAPL
 W+BLMR2         TLEWAMASLLKSPEVLKKAKAEIDDKIGHERLVDEPDILNLPYLQNIVSETFRLCPAAPL
                 ************************************************************

 Westar          LVPRSPSEDLKIGGYDIPRGTIVLVNSWAIHRDPRLWDEPERFMPERFEDKEAANNNKLM
 W+BLMR2         LVPRSPSEDLKIGGYDIPRGTIVLVNSWAIHRDPRLWDEPERFMPERFEDKEAANNNKLM
                 ************************************************************

 Westar          MFGNGRRTCPGAALGQRMVSLALGSLIQCFDWEKVNGEEIDMTENPGMAMRKLVPLRAVC
 W+BLMR2         MFGNGRRTCPGAALGQRMVSLALGSLIQCFDWEKVNGEEIDMTENPGMAMRKLVPLRAVC
                 ************************************************************

 Westar          HQRPIMTNLLA
 W+BLMR2         HQRPIMTNLLA
                 ***********
```

Supplemental Figure 4.2. Comparison in amino acid sequence of the CYP81F2 homolog between Westar and the developed lines containing *BLMR*2 (W+*BLMR2*). Note the amino acid change at aa 57 (in red). Zhang et al. (2021).



Supplemental Figure 4.3. HPLC report with glucosinolate peaks labeled based on previously reported order and results of 2.4.3.3.

Table 4.1 RT-qPCR data *CYP81F2-A10* in of Westar and three NIL, samples inoculated with *L. maculans* strain 87-41 or water. Actin used as reference gene, MM1 is query gene.

Sample	Time point	Treatment	RT-qPCR MM1	RT-qPCR Actin
HN7	2 DPI	*L. maculans* 87-41	27.01	22.08
			26.98	21.89
			27.02	21.78
			27.74	23.33
			27.42	23.03
			27.55	22.92
			28.58	21.52
			28.49	21.33
HN7	4 DPI	*L. maculans* 87-41	28.79	22.35
			29.14	22.25
			28.82	22.24
			26.18	21.55
			26.49	21.40
			26.01	21.19
			26.01	21.24
			26.09	20.93
			26.03	21.00
HN7	6 DPI	*L. maculans* 87-41	23.95	21.18
			24.60	20.85
			24.27	21.06
			24.91	21.04
			24.70	20.92
			24.49	21.15
			24.39	21.57
			24.73	21.47
			24.27	21.57
HN7	8 DPI	*L. maculans* 87-41	26.98	21.50
			26.96	21.30
			26.94	21.38
			25.01	21.10
			25.05	20.69
			24.32	20.91
			26.42	22.12
			26.65	22.01
			25.48	22.01
NJ11	2 DPI	*L. maculans* 87-41	31.67	26.08
			30.53	26.02
			30.92	26.49
			28.69	22.17
			28.19	22.37
			28.64	22.60

			28.68	22.22
			28.76	22.22
			28.78	22.46
NJ11	4 DPI	*L. maculans* 87-41	26.80	21.71
			28.56	21.75
			26.50	22.14
			26.49	21.65
			26.11	21.39
			26.33	22.01
			26.88	21.49
			26.61	22.15
			26.89	21.55
NJ11	6 DPI	*L. maculans* 87-41	27.21	21.36
			26.38	21.86
			26.68	21.80
			28.25	21.81
			27.50	21.82
			27.61	21.75
			27.76	23.53
			27.25	22.57
			26.88	22.36
NJ11	8 DPI	*L. maculans* 87-41	26.30	24.12
			26.41	23.30
			25.81	23.52
			25.47	23.24
			26.16	22.53
			25.29	22.66
			26.01	23.15
			26.21	22.67
			25.25	22.49
NJ13	2 DPI	*L. maculans* 87-41	26.17	21.89
			27.14	21.28
			26.32	21.31
			27.29	22.22
			27.68	22.02
			27.0	21.88
			28.78	22.04
			29.46	21.30
			28.46	21.32
NJ13	4 DPI	*L. maculans* 87-41	27.20	22.63
			27.45	21.78
			26.60	21.66
			27.06	21.60
			26.54	21.50
			26.22	21.60
			27.18	21.88

			26.52	22.03
			26.68	21.76
NJ13	6 DPI	*L. maculans* 87-41	25.36	22.18
			24.66	21.95
			24.60	21.65
			22.87	21.95
			24.55	21.76
			23.71	21.59
			26.01	21.47
			25.64	21.39
			25.74	21.35
NJ13	8 DPI	*L. maculans* 87-41	24.95	21.34
			24.06	21.38
			23.53	21.13
			26.13	21.61
			25.47	21.77
			25.30	21.54
			26.54	21.98
			25.90	22.04
			25.97	21.95
Westar	2 DPI	*L. maculans* 87-41	28.71	22.57
			28.67	22.23
			28.25	22.14
			27.41	21.94
			27.26	21.70
			26.87	21.62
			26.79	23.28
			27.07	23.16
			27.02	22.98
Westar	4 DPI	*L. maculans* 87-41	26.40	21.42
			26.37	21.68
			26.35	21.22
			24.75	21.17
			24.74	20.92
			24.80	22.29
			25.41	21.99
			25.73	21.77
			25.59	21.79
Westar	6 DPI	*L. maculans* 87-41	25.57	21.79
			25.70	21.59
			26.04	21.71
			26.72	22.53
			26.05	22.18
			26.12	22.20
			26.99	21.81
			26.60	21.84

			26.62	21.75
Westar	8 DPI	*L. maculans* 87-41	24.05	21.66
			24.17	21.57
			24.28	21.45
			24.03	21.52
			24.19	21.39
			24.43	21.35
			24.03	21.60
			24.19	21.48
			24.43	21.42
HN7	2 DPI	H_2O	25.93	26.14
			25.16	25.85
			25.51	26.00
			25.39	25.29
			24.79	25.23
			25.46	25.66
HN7	4 DPI	H_2O	28.98	22.33
			28.10	22.20
			28.76	22.21
			28.69	22.52
			28.58	21.84
			28.46	21.16
			29.42	22.16
			29.61	21.70
			28.62	21.97
HN7	6 DPI	H_2O	29.07	19.33
			29.82	18.85
			28.32	18.61
			28.49	21.57
			28.74	21.45
			28.99	21.68
			28.31	21.97
			28.21	21.60
			28.26	21.75
HN7	8 DPI	H_2O	28.20	23.37
			28.80	22.88
			28.62	23.03
			29.25	23.34
			28.22	23.01
			28.41	23.11
NJ11	2 DPI	H_2O	27.20	22.30
			27.17	22.21
			27.20	22.39
			27.91	22.31
			27.76	22.13
			27.31	22.12

			28.12	22.46
			26.82	22.18
			26.33	22.14
			28.68	21.82
			28.53	21.52
			28.32	21.45
			26.73	22.08
NJ11	4 DPI	H_2O	27.17	21.78
			26.81	21.72
			28.34	22.08
			28.92	21.92
			28.64	21.65
			28.53	22.37
			28.43	22.07
			28.33	22.20
			27.06	21.80
NJ11	6 DPI	H_2O	27.01	21.63
			26.96	21.56
			28.07	22.27
			27.54	22.12
			27.83	22.06
			27.64	21.06
			27.09	21.05
			27.76	21.00
			27.28	21.96
NJ11	8 DPI	H_2O	27.10	21.89
			27.34	21.96
			29.64	21.72
			28.81	21.68
			28.83	22.08
			28.08	21.82
			27.64	21.89
			27.84	21.78
			27.68	21.79
NJ13	2 DPI	H_2O	27.69	21.91
			27.90	22.01
			27.19	21.99
			27.41	21.91
			27.07	22.10
			29.21	23.24
			29.40	23.35
			29.43	23.34
NJ13	4 DPI	H_2O	26.14	22.04
			26.32	21.87
			26.63	22.22
			26.94	22.52

			26.44	22.51
			26.41	22.98
NJ13	6 DPI	H_2O	27.42	24.11
			27.53	24.12
			28.23	25.22
			26.60	22.13
			26.96	21.81
			26.66	22.39
NJ13	8 DPI	H_2O	27.17	22.04
			27.00	21.86
			26.94	23.23
			27.81	22.48
			27.65	22.49
			27.35	23.25
			26.54	21.43
			26.64	21.29
			26.45	21.38
Westar	2 DPI	H_2O	28.44	23.08
			28.48	23.30
			28.05	22.85
			27.07	21.98
			27.98	21.94
			27.30	22.02
			26.77	21.58
			26.89	21.60
			26.40	21.55
Westar	4 DPI	H_2O	27.54	21.37
			27.95	21.32
			27.26	21.42
			27.90	21.32
			27.70	21.40
			27.18	21.24
			28.00	20.98
			27.78	20.98
			27.51	20.98
Westar	6 DPI	H_2O	29.53	21.62
			29.44	21.70
			29.56	21.55
			27.94	22.74
			27.72	21.83
			27.34	23.65
			29.00	21.71
			28.11	21.59
			28.43	21.90
Westar	8 DPI	H_2O	27.41	21.28
			27.01	21.28

27.04	21.36
26.72	21.10
26.17	21.08
26.21	21.24
29.66	22.09
27.31	21.97
27.93	22.28

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