The Effect of Light and Temperature on Glucosinolate Concentration in Turnip (*Brassica rapa)*

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Veronica Lea Justen

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Dedication

This dissertation is dedicated to Ben. Thank you for your love and encouragement.

Abstract

Glucosinolates (GSLs) are thioglucosides produced by turnip (*Brassica rapa* subsp. *rapa)* and other cruciferous vegetables with important properties for plant defense and human health, particularly cancer prevention. We compared yield and GSL concentrations in the roots and shoots of turnip cultivars grown using plastic colored mulch and photoselective netting treatments in both a May and August planting. Turnip yield and GSL concentrations were dependent on tissue type, genotype and environmental factors. 'Just Right' roots and shoots consistently had the highest yield and gluconapin concentration of the cultivars examined in field experiments. In most instances, 'Scarlet Queen' roots had the highest total GSL concentrations while 'Just Right' roots had the least. Although colored plastic mulches significantly influenced both total and individual GSL concentrations, mulch-dependent increases in GSL concentrations were not consistent across tissue types, cultivars, planting dates and years of the study. Photoselective nettings did not consistently affect shoot or root yield or GSL concentration in root tissues. Netting was only a significant factor for glucobrassicanapin (GBN) concentration in shoots with the no netting treatment resulting in the highest GBN concentration. Planting date and year interactions were significant for total and individual GSL concentrations and proportions in both plastic mulch and photoselective netting experiments. These interactions are partially explained by differences in air temperatures and solar radiation prior to harvest. To assess the specific role of temperature on GSL concentration and biosynthetic regulation in roots and shoots, turnips were grown under three different temperature regimes in a controlled environment. Gene expression analyses indicate that some BrMYB transcription factor transcript levels are associated with temperature-dependent changes in GSL concentration, however this association varies between cultivar and tissue type. When compared to low temperature treatments, high temperature treatments increased total, aliphatic and indolic GSLs in a tissue and genotype specific manner. Gluconasturtiin (GNS), an aromatic GSL, concentration was inversely correlated with temperature with high temperature treatments resulting in 20% and 48% less GNS than low temperature treatments in JR and SQ roots, respectively. The indolic GSL, 1-methoxyglucobrassicin (1MGB) was the root GSL most elevated by increased temperature resulting in a nine-fold increase on average in both cultivars between the low and high temperature treatments. These results show promise for the use of temperature to enhance the health promoting properties of turnip as 1MGB has potent chemopreventive effects.

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1 Literature Review

1.1 Introduction

Glucosinolates are secondary metabolites characteristic of the order Brassicales whose hydrolysis products play a role in plant defense and have potential for human health. Glucosinolate concentrations and profiles vary between plant tissues and genotypes, and accumulation is highly subject to environmental conditions. Turnip is a cruciferous vegetable with edible portions both above and below ground, making it a unique crop for examining the role of the environment on glucosinolate accumulation in different tissues. Defining and manipulating cultural practices to maximize glucosinolate concentration in vegetable tissues could have significant impacts on human health and the nutraceutical industry. Here we summarize the current state of knowledge regarding the environmental and genetic control of glucosinolates in cruciferous vegetables and the model plant *Arabidopsis*.

1.2 Turnip

Turnip is a unique crop as it has edible portions both above and below ground; both turnip greens and roots have culinary uses either raw or cooked. Turnip roots provide a good source of vitamin C, calcium and iron (USDA 2009). Aside from its use for human consumption, turnip has been valued for centuries as a forage crop.

Center of origin and phylogeny

Turnip, a diploid species in the Brassicaceae, has a chromosome number of 2n=2x=20. Turnip most likely has a center of origin in Europe (DeCandolle 1886) and is recorded as being planted in the United States as early as 1540 (Hedrick 1919). Turnip is a subspecies (*rapa*) within *Brassica rapa*, which includes other vegetables such as Chinese cabbage (*pekinensis* group) and bok choy (*chinensis* group). *Brassica rapa* has a unique capability to intercross with subspecies within *Brassica oleracea* (n=9) and *Brassica nigra* (n=8). Crosses with these species result in *Brassica napus* (n=19) and *Brassica juncea* (n=18), respectively.

Production

Although grown for the fresh market as a short season crop, turnip is actually a biennial and requires a vernalization period of only three weeks with mean temperatures less than 50°F to induce flowering, or bolting. Turnips can be planted in the spring or fall, producing greens approximately four weeks after planting and mature roots (2-3" in diameter) between 50-70 days after planting. Turnips are typically seeded then thinned to a distance of 2" between plants to allow for uniform root development. As with most root crops, turnips require consistent soil moisture throughout the growing season to ensure uniform root development and to prevent root cracking. Turnips are also sensitive to iron, calcium and boron deficiencies. Boron deficiencies can be the most serious deficiency causing necrosis and resulting in hollow roots.

Turnips are sensitive to post-harvest conditions. Although they are resistant to damage from freezing and chilling, both greens and roots require proper storage conditions of 32°F and 95-100% relative humidity (Nunes and Emond 2003). In such conditions, turnips have a shelf-life of 10-14 days when bunched and 4-6 months when topped. Coatings with edible waxes are also used to retard water loss in roots.

1.3 Glucosinolates

Introduction

Glucosinolates (GSLs) are a unique group of secondary metabolites characteristic of plants in the order Brassicales. The general structure of glucosinolates is a glucose bound to a modified amino acid via a thioester bond (Figure 1.1). Three general groups of GSLs exist: aromatic GSLs derived from phenylalanine or tyrosine; indolic GSLs derived from tryptophan; and aliphatic GSLs derived from methionine, isoleucine, leucine, alanine, or valine. Approximately 120 GSLs have been identified (Fahey et al., 2001). Table 1.1 provides a list of the common and chemical name of the major GSLs found in cruciferous vegetables and their classified R group.

GSLs react with a thioglucosidase, myrosinase (thioglucoside glucohydrolase, EC 3.2.1.147), that hydrolyzes the thioester bond releasing glucose and creating unstable by-

products such as isothiocyanates. These hydrolysis by-products have significant values for human health, plant defense, and biofumigation in other agricultural crops.

Glucosinolates in Human Health and Plant Defense

GSLs are of interest to the medical community as their hydrolysis by-products provide potential health benefits. The hydrolysis of GSLs by myrosinase (ß-glucosidase) produces a combination of isothiocyanates, nitriles and thiocyanates and other by-products depending on reaction variables such as pH, the presence of ascorbic acid (Bones and Rossiter, 1996) and the activity of epithiospecifier proteins (Ute and Burow, 2007). GLSs are generally sequestered in the vacuole and myrosinase is localized to specific myrosin cells (Bones and Rossiter, 1996). As myrosinase is physically separated from GSLs in intact cells, the hydrolysis reaction occurs in great amounts only after cell disruption.

Absorption of isothiocyanates by animals induces enzymes in the liver involved in blocking carcinogen activation (phase I enzyme inhibition), detoxification (phase II enzyme activation) and apoptosis (Hecht, 2000; Kuem et al., 2004; Munday and Munday, 2004; Smith et al., 2003; Talalay and Fahey, 2001). Studies have implicated an important role for raw cruciferous vegetables in the diet in decreasing the onset of many cancers including lung and stomach (Hecht et al., 2004; Lynn et al., 2006; Tanaka et al., 2006). The isothiocyanate derivatives of only a few of the 120 known glucosinolates have been extensively studied for their impact on human health. The glucobrassicin-derived isothiocyanate, indole-3-carbinol; 1-methoxyglucobrassicin-derived, N-methoxyindole-3-carbinol; gluconasturtiin-derived, phenylethyl isothiocyanate; and glucoraphanin-dervied isothiocyanate, sulphoraphane, have been specifically studied for their effects on cancer prevention (*e.g.* Neave et al., 2005; Pham et al., 2004; Plate and Gallaher, 2006). 1-methoxyglucobrassicin and gluconasturtiin are major GSLs in turnip root tissues (Smetanska et al., 2007).

Although some GSL-derived isothiocyanates have beneficial human health impacts, GSLs can have undesirable characteristics for animals. Many isothiocyanates impart a characteristic taste and olfactory sensation which influence consumer acceptance

of produce (Engel et al., 2006). Isothiocyanates from some Brassicaceae products such as wasabi, horseradish, and mustard can also elicit a pain response by stimulating nerve receptors (Jordt et al., 2004). GSL degradation products, particularly from hydroxyalkenyl GSLs such as progoitrin, can cause a suite of animal health problems including reduced intake, iodine deficiency and thyroid and kidney hypertrophy (Tripathi and Mishra, 2007). The negative effects of GSLs on animal health have led to breeding efforts to decrease GSL content in agronomic crops. One result of such breeding efforts is canola, a rapeseed low in erucic acid and GSLs.

GSL hydrolysis by-products also play a significant role in plant defense. They can both deter (Agrawal and Kurashige, 2003) and attract various feeding insects (Mewis et al., 2002). GSL content in plant tissues can also be negatively correlated with insect performance (Mewis et al., 2006). GSL biosynthesis may be induced by mechanical injury, disease pressure, or injury signals such as jasmonic acid (JA), salicylic acid (SA), and ethylene (Jost et al., 2005). Indolic glucosinolate biosynthetic genes have been correlated with exposure to *Erwinia carotovora* (*CYP79B3*), *Psuedomonas syringae* (*CYP79B2*) and wounding (*CYP79B2*) (Brader et al., 2001). These genes code for enzymes which convert tryptophan into indole-3-acetaldoxime. Methoxylation of GSLs can be influenced by different elicitors: 1-methoxyglucobrassicin biosynthesis is induced by JA and inhibited by ethylene precursors while 4-methoxyglucobrassicin is induced by ethylene precursors and SA homologs, but inhibited by JA (Mikkelsen et al., 2003).

Biosynthetic and Regulatory Genes

The GSL biosynthetic pathway has been the focus of several reviews (*e.g.* Grubb and Abel, 2006). An outline of GSL biosynthesis including the biosynthetic and regulatory genes involved is outlined in Figure 1.2. As described previously, GSLs are derived from amino acids. In the case of indolic GSL biosynthesis, tryptophan is converted to indole-3-acetaldoxime (IAOx) in the endoplasmic reticulum via a cytochrome P_{450} enzyme (*CYP79B2/3;* Hull et al., 2000; Mikkelsen et al., 2000). IAOx is the biosynthetic branching point between camalexins (indole alkaloids), indolic GSLs, and indole-3-acetic acid. Further modification of IAOx into indolic GSLs is carried out

via several enzymes in the cytoplasm. Another cytochrome P_{450} enzyme (*CYP83B1*, Bak et al., 1998; Hansen et al., 2001a; Naur et al., 2003a) and C-S lyase result in the conversion of the aldoxime into thiohydroxamic acid (Naur et al., 2003b). A thio-glucosyltransferase (*UGT74B1)* converts thiohydroxamic acid into desulfo-GSL (Grubb et al., 2004). Sulfotransferase enzymes (*STa* for indolic and aliphatic and *STb/c* for aliphatic) then convert the desulfo-GSL into the GSL, which can be stored in the vacuole (Piotrowski et al., 2004). The production of the core GSL structure of aliphatic and aromatic GSLs is similar to that described for indolic GSLs with *CYP79F1/2* and *CYP79A2* activity resulting in aliphatic and aromatic aldoxime formation, respectively (Hansen et al., 2001b; Chen et al., 2003); and *CYP83A1* and *CYP83B1* oxidizing the aliphatic and aromatic aldoxime, respectively (as reviewed by Grubb and Abel, 2006).

Much of the diversity within the glucosinolate profile of plant tissues arises from the various aliphatic glucosinolates that can be created from methionine side chain elongation and modification. Methionine side chain elongation occurs prior to the GSL core structure biosynthesis and involves several steps including a condensation reaction performed by methylthioalkylmalate synthases (*MAM1*, *MAM2*, and *MAM3*; Kroymann et al., 2001; Textor et al., 2007). *MAM3* has been shown to be involved in the addition of up to eight carbons to the methionine precursor (Textor et al., 2007). After the biosynthesis of the core GSL molecule, aliphatic GSLs may undergo additional stepwise modifications including oxidation of the methylthio sulfur via flavin monooxygenase, conversion of the subsequent methylsulfinyl moiety into an alkenyl group via AOP (2-oxoglutarate-dependent dioxygenase; Kliebenstein et al., 2001b), hydroxylation of the alkenyl group and finally conjugation of the hydroxyalkenyl group to benzoic acid (Halkier and Greshenzon, 2006; Kliebenstein et al., 2007).

Both aliphatic and indolic GSL biosynthesis are regulated by various genetic components. In *Arabidopsis*, these regulatory elements can either repress GSL production, such as *SLIM1* (*Sulfur Limitation1*; Maruyama-Nakashita et al., 2007), promote GSL production such as *AtDof1.1* (DNA-binding-with-one finger; Skirycz et al., 2006) and subgroup 12 R2-R3 MYB transcription factors (Gigolashvili et al., 2009); or

both positively and negatively regulate GSL production such as the nuclear-localized calmodulin-binding protein IQD1 (Levy et al., 2005). Two distinct clades of subgroup 12 R2-R3 MYB transcription factors regulate GSL biosynthesis and accumulation. Clade 1 members *MYB34*, *MYB51* and *MYB122* regulate indolic GSL biosynthesis while clade 2 members *MYB28*, *MYB29* and *MYB76* regulate aliphatic GSL biosynthesis (Figure 1.2; Gigolashvili et al., 2009). Members within each clade appear to have distinct, but overlapping roles. It has been suggested that *MYB34*, *MYB122* and *MYB 51* positively regulate glucobrassicin, but only *MYB51* positively regulates other GSLs such as methoxylated indolic glucosinolates (Gigolashvili et al., 2007). However, Malitsky and colleagues (2008) did not observe this trend and found that both *MYB34* and *MYB51* overexpression resulted in increases in 1-methoxyglucobrassicin.

MYB28 regulates both long (6-8 carbon length) and short-chain (3-5 carbon length) aliphatic GSL biosynthesis and its overexpression can result in increased accumulation of indolic GSLs (Hirai et al., 2007). *MYB29* and *MYB76* also regulate GSL biosynthesis to a lesser degree, but only short-chain aliphatic GSL biosynthesis (Sønderby et al., 2007; Gigolashvili et al., 2008). MYB transcription factors may also regulate GSL biosynthesis in a tissue-specific manner with *MYB34* and *MYB122* being preferentially expressed in root tissues and *MYB51* being preferentially expressed in leaf tissue (Gigolashvili et al., 2009). *MYB28*, *MYB29* and *MYB76* appear to be expressed to some degree in all tissues (Gigolashvili et al., 2009). In most cases, the expression pattern of MYB transcription factors correlates with expression of other GSL biosynthetic genes (Gigolashvili et al., 2009). No MYB transcription factors have been identified that specifically regulate aromatic GSL biosynthesis.

Using a comparative genomics approach, Zang and colleagues (2009) identified GSL biosynthetic and regulatory orthologs in *Brassica rapa* (Br) with high sequence identity (72-94%) to *Arabidopsis thaliana* GSL biosynthetic genes. In most cases, multiple copies of these genes were found in the *B. rapa*. Two MYB transcription factor orthologs, *MYB76* and *MYB122*, were absent or non-functional in *B.rapa*. Br*MYB122-1* was found to be non-functional due to a deletion that resulted in a premature stop codon

while *MYB76* was absent in *B.rapa* (Zang et al., 2009). The absence of *MYB76* in *B. rapa* is interesting because *MYB76* positively regulates methylsulfinylated short-chained aliphatic GLSs in *Arabidopsis* (Sønderby et al., 2010) and these GLSs are absent or in low concentrations in turnip (Carlson et al., 1987; Li et al., 2007; Smetanska et al., 2007).

1.4 Factors affecting glucosinolate accumulation in plants

The types and amounts of glucosinolates found in a given tissue can be influenced by a number of factors, resulting in unique profiles depending on genotype by environment interactions. Few studies have provided information about environmental effects on GSL profile in turnip, particularly the differential effects of environmental factors on shoot and root GSL profiles. GSLs are associated with stress responses and thus have been studied with respect to their production in environments such as those with water stress (Schreiner et al., 2009), herbivory (De Villena et al., 2007), pathogen attack (Brader et al, 2001) and nutrient deficiencies (Rosen et al., 2005). Below we outline the current state of knowledge of how genotype, tissue, temperature, and light influence GSL concentrations.

Genotype

Glucosinolate profiles vary widely among members of the order Brassicales with some species producing mostly one type of glucosinolate (i.e. *Nasturtium officinale* primarily accumulates the aromatic GSL gluconasturtiin; Kopsell et al., 2007) and other species producing mixtures (i.e. cabbage accumulates both aliphatic and indolic GSLs; Rosen et al., 2005). Glucosinolate profiles also vary within a species both among different cultivars and within breeding lines. Carlson et al. (1987) assessed the GSL variation of 14 turnip cultivars used for animal feed, greens and root consumption and identified distinct differences in indolic, aliphatic and aromatic GSL accumulation among the cultivars examined. A survey of 39 *Arabidopsis* ecotypes showed that diverse glucosinolate profiles exist within the species, particularly with respect to aliphatic GSL accumulation (Kliebenstein et al., 2001a). Much research has been done to assess the GSL variation among broccoli genotypes. Efforts have been made to introgress wild broccoli genes into commercial cultivars to increase glucoraphanin concentrations in

florets. This research has identified one quantitative trait locus that accounts for glucoraphanin variation, in some cases up to a threefold difference, observed between broccoli genotypes (Sarikamis et al., 2006).

Tissue-type

Recently, van Dam and colleagues (2009) conducted a meta-analysis comparing root and shoot GSL profile data from 29 species including turnip. Their analysis highlighted several differences between root and shoot GSL profiles. In general root tissues have significantly higher GSL concentrations than shoots (4-5 times more on average). Root tissues tend to have significantly higher concentrations of the aromatic GSL, gluconasturtiin (GNS), and methoxylated indolic GSLs than shoot tissues. Carlson and colleagues (1987) found that the aliphatic GSLs glucobrassicanapin (GBN) and gluconapin (GNP) were the predominant GSLs in turnip shoot tissues. Smetanska et al. (2007) also found GNP to be the predominant shoot GSL in turnip shoot tissue. Zhang et al. (2008) found that GNS dominated the GSL profile in turnip root tissues while Smetanska et al. (2007) found 1-methoxyglucobrassicin (1MGB) to be the primary GSL in turnip root tissues. Tissue-dependent differences in turnip GSL profiles may be the result of differential regulation of GSL biosynthesis between the tissues, which has been suggested to occur in the *Arabidopsis* accession Columbia (Hirai et al., 2007).

GSL concentrations can vary even within a tissue type. Nilsson and colleagues (2006) found significant GSL variation between concentric tissues of the cabbage head, with sinigrin concentrations increasing from inner to middle to outer regions and glucobrassicin concentrations increasing from middle to outer to inner regions of the cabbage head. Shroff and colleagues (2008) found that aliphatic and indolic GSLs have a non-uniform distribution within *Arabidopsis* leaf tissue with glucoraphanin having the highest concentration in the midvein and glucobrassicin having the highest concentration in the outer lamina. The different distribution of the glucosinolates within tissues supports previous suggestions that individual GSLs are under differential regulation (Gigolashvili et al., 2009).

Temperature and light quantity

The influence of climatic factors associated with different planting dates on GSL concentrations has been extensively studied in cruciferous vegetables such as cabbage (Nilsson et al., 2006), broccoli (Vallejo et al., 2003) and kale (Cartea et al., 2007). Several studies have measured differences in GSL concentrations of different cabbage varieties across growing seasons. Nilsson and colleagues (2006) surveyed GSL levels in several cabbage varieties grown over a two-year period. Changes in glucobrassicin concentrations were the greatest contributors to changes in total GSL levels which varied drastically between the two years of the study. The authors attributed the increase in glucobrassicin during the second year to an increase of 1°C in average temperature. Charron et al. (2005a) reported genotype by environment interactions for cabbage GSL concentrations with green cabbage having higher GSL content than red cabbage in spring plantings but lower GSL levels than red cabbage GSLs in fall plantings. Charron et al. (2005b) also found that myrosinase activity is influenced by environmental conditions; with activity being generally higher in fall-planted than in spring-planted vegetables. The authors attributed the seasonal increase in myrosinase activity to higher mean temperatures and lower photosynthetic photon flux. Charron and Sams (2004) found that the concentration of the aliphatic GSL, GNP, doubled in leaves and stems of *B. oleracea* plants grown at 32 °C and the indolic GSL, 1MGB, increased three-fold in roots grown at 32 °C compared to those grown at 12 °C. The authors also examined simulated spring and fall growing conditions in a controlled environment and determined that the effects of temperature and light quantity from the simulated growing seasons were not additive but worked interactively to alter GSL concentrations (Charron and Sams, 2004). Engelen-Eigles and colleagues (2006) found that the concentration of the aromatic GSL gluconasturtiin was increased in *Nasturtium officinale* grown at 10 °C and 15 °C as compared to that grown at 20 °C or 25 °C.

Light quality

Several studies suggest that light quality can influence GSL content. Antonious et al. (1996) found that turnips grown with different colored plastic mulches (black plastic painted blue, white or green) resulted in differential increases in total GSLs. In this study, the blue mulch treatment resulted in the highest concentrations of total GSLs, which the authors attributed to the higher amount of blue light reflected by the blue mulch treatment (Antonious et al., 1996). Individual GSL concentrations were not examined in this study. Engelen-Eigles et al. (2006) concluded that a continuous red light treatment in a growth chamber over a 2-week period can increase gluconasturtiin, an aromatic GSL, in watercress (*Nasturtium officinale*) by 39% as compared to metal halide and far-red light treatments. End of day red light treatments also significantly increased gluconasturtiin levels.

Expression of genes involved in glucosinolate biosynthesis can also be influenced by light quality. Hoecker et al. (2004) found that continuous red light for 3 days increased *CYP83B1* transcript levels in *A. thaliana* seedlings. The effect was similar for *Arabidopsis* phytochrome B (*phyB*) mutants, suggesting that the overexpression of *CYP83B1* is not *phyB* mediated. Continuous far-red light had no effect on *CYP83B1* transcript levels (Hoecker et al., 2004). Therefore, increases in *CYP83B1* expression, due to changes in light quality may result in an increase in indolic GSL concenctrations as *CYP83B1* has been shown to be involved in indolic GSL biosynthesis (Hansen et al., 2001a).

1.5 Altering light in agricultural production systems

Plastic colored mulches

Colored mulches provide a means to alter soil temperature and the light quality reflected onto a plant. Colored mulches are thin plastic films laid tightly over the soil surface surrounding crop plants. They range in thicknesses between 1-2 mil (0.025-0.05mm), and generally come in widths of 1.22-1.52 m. The color of colored mulches can be consistent throughout the thickness of the mulch or co-extruded, with one color overlaid on another. Colored mulches can also be produced to have infra-red (or

selective light) transmitting properties by incorporating special pigmentation into the plastic that allows maximum infra-red transmission to the soil surface while minimizing visible light transmission. Studies have shown that plastic colored mulches influence the photosynthetic photon flux, and amount of red, far-red and blue light reflected onto crops in the field, providing a means to influence processes regulated by phytochrome or cryptochrome mechanisms, respectively (Kasperbauer 1992). They have been used to alter secondary metabolite and flavor compound concentration in several crops including basil, strawberry and turnip (Antonious et al., 1996; Atkinson et al., 2006; Loughrin and Kasperbauer, 2001).

Photoselective nettings

One way to alter light quantity and light quality in a field setting is by using photoselective nettings. These nets have the ability to influence the amount and spectra of radiation transmitted to crops, but also can influence environmental conditions such as relative humidity, temperature and air movement (as reviewed by Stamps, 2009). Photoselective nettings have been used in many crops to alter vegetative growth, flowering and crop yield and quality. Nettings have mostly been used with floral, ornamental foliage (Stamps, 2008) and fruit crops (Retamales et al., 2008), however, some research has been done to assess the use of netting in bell pepper (*Capsicum annuum*) production (Elad et al., 2007; Fallik et al., 2008). Elad and colleagues (2007) found that nettings increased yield and fruit quality in bell peppers when compared to a no netting control. No information on the use of photoselective nettings to alter secondary metabolites such as GSLs is available.

1.6 Conclusions

GSL biosynthesis and accumulation varies among genotypes and can be influenced by many environmental factors including light and temperature. Because GSL biosynthetic regulation and accumulation differ between root and shoot tissues, turnips provide a uniquely well-suited crop to examine the differential effect of environmental factors on root and shoot GSL concentrations. Better understanding of the tissue-specific and environmentally-dependent changes in GSL concentrations of different turnip

genotypes will provide useful information to maximize the health potential of cruciferous vegetables.

Table 1.1 Glucosinolates commonly found in cruciferous vegetables.

Common name	Chemical name	R-group
Glucoraphanin (GR)	4 (methylsulfinyl)-butyl	Aliphatic
Progoitrin (PRO)	2-hydroxy-3-butenyl	Aliphatic
Gluconapoleiferin (GF)	2-hydroxy-4-pentenyl	Aliphatic
Sinigrin (SN)	2-propenyl	Aliphatic
Gluconapin (GNP)	3-butenyl	Aliphatic
Glucobrassicanapin (GBN)	4-pentenyl	Aliphatic
1-methoxyglucobrassicin (1MGB)	1-methoxy-3-ylmethyl	Indolic
Glucobrassicin (GB)	Indol-3-ylmethyl	Indolic
4-methoxy glucobrassicin (4MGB)	4-methoxy-3-ylmethyl	Indolic
4-hydroxy glucobrassicin (4OGB)	4-hydroxyindol-3-ylmethyl	Indolic
Gluconasturtiin (GNS)	2-phenylethyl	Aromatic

Figure 1.1 General glucosinolate structure.

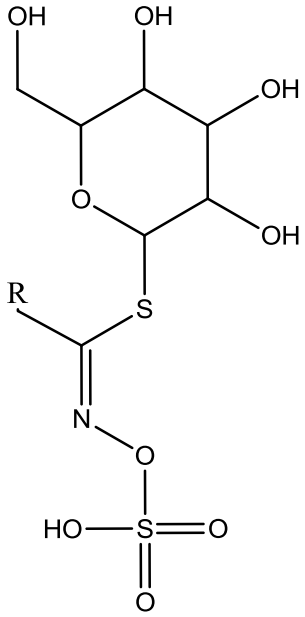
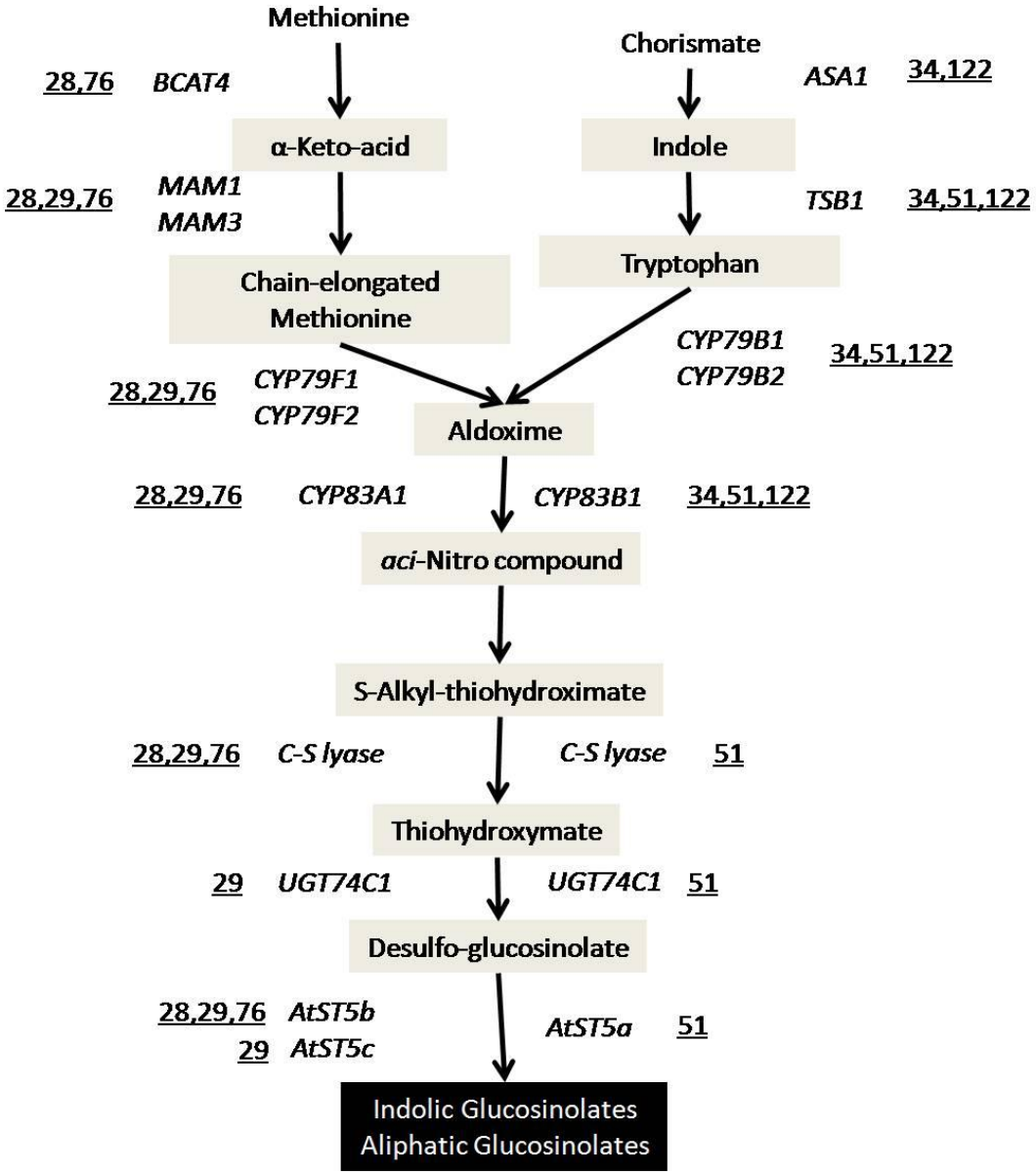


Figure 1.2 Schematic representation of the glucosinolate biosynthetic pathway including regulation by group 12 R2R3-MYB transcription factors. Glucosinolate intermediates (gray boxes) and end products (black box) are indicated. Enzymes required for each step are shown in italicized uppercase letters. Underlined numbers indicate activation of biosynthetic enzymes by *MYB29*, *MYB28* and *MYB76* (29, 28, and 76, respectively) for aliphatic GSLs or activation by *MYB34*, *MYB51* and *MYB122* (34, 51, and 122, respectively) for indolic GSLs. (adapted from Gigolashvili et al., 2009).



2 Seasonal variation in glucosinolate accumulation in turnip cultivars grown with colored plastic mulches

2.1 Summary

Glucosinolates (GSLs) are thioglucosides with important properties for plant defense and human health. GSLs are influenced by environmental factors such as light quality, temperature and planting date. Colored plastic mulches can influence reflected light quality and soil temperature. The objective of this study was to quantify yield and GSL concentration in turnip (*Brassica rapa* subsp. *rapa)* roots and shoots grown using colored plastic mulches. Four turnip cultivars ('Just Right', 'Purple Top', 'Royal Crown', and 'Scarlet Queen') were grown over five mulch treatments: white, yellow, silver, red, blue and a no mulch control in both a May and August planting in 2006 and 2007. 'Just Right' turnips had the highest shoot and root yield in both planting dates and years of the study, however there was no consistent relationship between mulch treatment and shoot or root yield for both planting dates. GSL concentrations and profiles varied with tissue type, genotype, and environmental factors. 'Just Right' roots and shoots consistently had the highest gluconapin (3-butenyl GSL) concentrations of the cultivars examined. In most instances, 'Scarlet Queen' roots had the highest total GSL concentrations while 'Just Right' roots had the least. Although mulch significantly influenced both total and individual GSL concentrations, mulch-dependent increases in GSL concentrations were not consistent across tissue types, cultivars, planting dates and years of the study. The inconsistency of mulch-dependent GSL enhancement may be due to differences in climatic factors and mulch-dependent changes in soil temperature between planting dates and years of the study.

2.2 Introduction

Glucosinolates (GSLs) are thioglucosides found in *Brassica sp.* vegetables that when hydrolyzed at the S-glucose bond create a suite of products involved in plant defense, flavor and human health. GSLs react with myrosinase (thioglucoside glucohydrolase, EC 3.2.1.147), that hydrolyzes the thioester bond releasing glucose and

creating unstable by-products such as isothiocyanates, thiocyanates and nitriles (Bones and Rossiter, 1996). These hydrolysis by-products have significant values for human health, plant defense, and biofumigation in the production of other agricultural crops. Identifying vegetable production systems that enhance GSL concentration and myrosinase activity could have a significant impact on human health, particularly cancer prevention, as GSL hydrolysis products have potent chemopreventive benefits (Neave et al., 2005; Pham et al., 2004; Plate and Gallaher, 2006).

GSL concentration is influenced by genotype and environmental factors including temperature and light quality. Light quality and temperature can be altered in a field setting by changing planting date (Charron and Sams, 2004; Charron and Sams, 2005) and by growing plants with colored plastic mulches (Antonious et al., 1996). Several studies have shown that colored plastic mulches influence the photosynthetic photon flux, and amount of red, far-red and blue light reflected onto crops in the field, providing a means to influence processes regulated by phytochrome or cryptochrome mechanisms, respectively (Kasperbauer, 1992). Colored plastic mulches have been used to alter secondary metabolite concentrations and flavor profiles in several crops including basil and strawberry (Loughrin and Kasperbauer, 2001; Atkinson et al., 2006). Antonious et al. (1996) found that turnips grown with different colored mulches (black plastic mulch painted blue, white or green) resulted in differential increases in total GSLs. In this study, the blue mulch treatment resulted in the highest concentrations of total GSLs, which the authors attributed to the higher amount of blue light reflected by the blue mulch treatment (Antonious et al., 1996). This study did not examine the influence of colored plastic mulches on individual GSL concentrations or on shoot GSLs. Understanding the effect of field treatments on the GSL profile of shoot and root tissues rather than just total GSL concentration in roots is important as shoots and roots inherently have different GSL profiles (as reviewed by vanDam et al., 2009) and individual GSLs have different effects on plant defense and human health (Neave et al., 2005; Plate and Gallagher, 2006). Additionally, regulation of GSL biosynthesis may occur in a tissue-specific manner (Hirai et al., 2007).

GSL profiles differ between *Brassica* species (Mithen et al., 1987) and cultivars within a species (Carlson et al., 1987; Rosen et al., 2005). GSL variation within a species may be correlated with anthocyanin accumulation as previous research has suggested that red cabbage has higher GSL concentrations than green cabbage (Rosen et al., 2005) and purple cauliflower florets have higher sinigrin concentrations than white or green florets (Volden et al., 2009). We wanted to better understand the effect of reflected light quality and soil temperature on yield and GSL accumulation in vegetable tissues both above and below the soil surface. In this study we quantified yield and both total and individual GSL concentrations in turnip roots and shoots of four cultivars grown with different colored plastic mulches at two planting dates. This set of cultivars was used to provide a range of root pigmentation.

2.3 Materials and Methods

The study was conducted at the Southern Research and Outreach Center in Waseca, Minn. (44.07° N , 93.52°W) on a Webster Clay loam soil (fine-loamy, mixed superactive typic endoaquoll with a pH of 6.8 and soil P and K of 40 ppm and 158 ppm, respectively) utilizing a randomized complete block design with four replications. Plots consisted of 7.6m rows on raised beds spaced at 1.5m on center. Plots were fertilized with 67kg/ha N pre-plant. Planting dates were 6 May and 7 August 2006 and 10 May and 28 August 2007. The trial included four turnip cultivars: 'Just Right'(JR; Jordan's Seed, Woodbury, MN), 'Purple Top'(PT; Jordan's Seed, Woodbury, MN), 'Royal Crown'(RC; Sakata Seed America, Inc., Morgan Hill, CA) and 'Scarlet Queen' (SQ; Johnny's Seeds, Winslow, ME). These genotypes were chosen based on their pigmentation. JR has a white skin, PT and RC are mostly white skinned with purple skin at the top of the root and SQ has an entirely red skin. SQ shoots also have red petioles and venation. JR, RC and SQ are hybrid turnip cultivars, while PT is open-pollinated.

Turnip cultivars were grown with six different mulch treatments: yellow (Yellow/SLT, Ginegar Plastic Products, Ltd., Israel), blue, red, white, silver (Jordan's Seed, Woodbury, MN), and a no mulch control. Blue and red mulches were infra-red transmitting plastic mulch films while yellow, silver and white were co-extruded plastic

mulch films. Mulches were chosen based on their spectral properties (Table 2.1; Figure S1). Light quantities reflected by mulches were measured using an Apogee Model Spec-UV/PAR spectroradiometer with reflectance probe attachment (Apogee Instruments, Inc., Logan,UT). Reflected red to far-red light ratios (R: Fr) were measured with a Skye red-far red meter (Skye Instruments, Ltd., Powys, UK).

Turnip seeds were planted with 5cm spacing between seeds using a two-row Nibex 500 Precision Planter (Emeryville, CA). Plastic mulches were installed immediately following seeding using a commercial plastic mulch layer (Mechanical Transplanter Co., Holland, MI). Plastic mulches were then slit to allow seedling emergence through the mulch surface as depicted in Figure S2. Plots were irrigated as needed with drip irrigation placed under the mulch surface to provide a minimum of 2.5 cm of water each week. Conventional management practices were utilized for pest and weed management. Soil temperatures under each mulch type were monitored 5cm below the soil surface for all planting dates using an Optic StowAway® data logger, model WTA08 (Onset Computer Corp., Pocasset, MA) (Table 2.2; Figure S3). Seasonal temperature and solar radiation data (Table 2.3) were obtained from the Southern Research and Outreach Center weather information website (http://sroc.cfans.umn.edu/WeatherInformation/index.htm).

Whole plants were harvested when root diameter was between 5.0-7.6 cm. Twenty plants were randomly harvested from each plot. Plants were harvested between 09:00-13:00h. Plants were topped, and shoot and root weights were recorded separately for yield measurements. A composite sample of four plants was used for GSL quantification. All samples were stored at 4°C prior to processing for GSL quantification and were processed within two weeks after harvest.

Glucosinolate quantification. Extraction and GSL quantification was performed as per Hecht et al. (2004), utilizing modifications from Rosen et al. (2005). Briefly, a 150g leaf sample or 200g root sample was boiled in 700 mL of boiling water for 10 min to deactivate myrosinase. Boiled samples were macerated in a blender for 2 min. A 40 mL-aliquot of blended sample was homogenized using a BioSpec M133 Homogenizer

(BioSpec Products, Inc., Bartlesville, OK) set at 12,000 rpm for 2 min, then centrifuged for 10 min at 5000 *g*, 4°C.

Desulfoglucosinolate (ds-GSL) extraction was performed using conditioned solid phase strong anion exchange (SAX) columns (Sigma-Aldrich, St. Louis, MO). Sinigrin (2-propenyl GSL; Sigma-Aldrich, St. Louis, MO) was added to the conditioned SAX columns as an internal standard. To desulfate, samples were incubated with two units (0.2mg/mL) of sulfatase (aryl-sulfate sulfohydrolase; EC 3.1.6.1; Sigma-Aldrich, St. Louis, MO) on SAX columns for ~15 hr at room temperature (~21°C) then eluted with 3mL water and the collected volume was determined by weight. Further washing of the columns yielded no additonal ds-GSLs confirming complete elution. Eluent was stored at -20°C until HPLC analysis.

HPLC analysis was performed on an Agilent 1200 Series Quaternary system (Agilent technologies, inc., Santa Clara, CA) set at λ=229 nm using a Luna C18, 5µm, 250 x 4.6 mm column (Phenomenex, Torrance, CA) set at 30ºC. A 50-µL aliquot of the eluent was separated on the system with a flow rate of 1.0mL·min^{-1} using the following gradient: solvent A= water and B= acetonitrile; 0 to 2 min, 95% A, 5%B; 2 to 20 min, 85% A, 15% B; 20 to 23min, 53% A, 47% B; 23 to 30 min, 0% A, 100% B; and 30 to 33 min, 95% A, 5% B. Peaks were integrated using ChemStation for LC 3D Systems, Rev. B.04.01 software. A sample chromatogram is presented in Figure S4. GSL peak identities were confirmed using retention time and Ultra Performance Liquid Chromatography-Mass Spectrometry (Waters Corporation, Milford, MA) using a C18 column, a water:acetontirile gradient and negative electrospray ionization (see Figure S5 for example mass spectra). Ds-GSL concentrations were calculated using relative quantification with an internal standard (sinigrin) and previously published response factors (EU, 1990). Ds-GSL concentrations are reported on a µmol·100g^{-1} fw basis.

Statistical analysis. Data were analyzed with R 2.9.1 (R Foundation for Statistical Computing, Vienna, Austria). The significance of differences between treatments, cultivar and years was assessed by a fixed-factor ANOVA. Mean values were considered significantly different at $P < 0.05$ as determined by Tukey's HSD.

2.4 Results and Discussion

Shoot Yield. Shoot yields differed by cultivar and were inconsistently influenced by mulch treatments across planting dates and years (Table 2.4). In May and August plantings, cultivar (C), mulch (M), year (Y) and several interactions between these factors significantly influenced shoot yield (Table 2.4). The C x Y interaction was significant for both planting dates; however, this interaction was due to changes in the magnitude of yield between 2006 and 2007. Despite the significance of the C x Y interaction, the rank of JR and SQ remained the same between years and planting dates with JR yielding the most shoot biomass (23 and 24 $Mg \cdot ha^{-1}$ for May and August plantings, respectively) and SQ the least (14 $Mg \cdot ha^{-1}$ for May and August plantings). PT and RC had intermediate yields in both years of the study. In May plantings silver and yellow mulch treatments resulted in higher shoot yields (19 $Mg \cdot ha^{-1}$) than red mulch treatments (17 $Mg \cdot ha^{-1}$; Table 2.4). In August plantings, no mulch control plots yielded more shoot biomass (21 $Mg \cdot ha^{-1}$) than all other mulch treatments.

Root Yield. Root yields differed by cultivar and were inconsistently influenced by mulch treatments across years and planting dates. In May plantings, C, M, and Y were significant factors influencing root yield as was the C x Y interaction (Table 2.4). The C x Y interaction for root yield in May plantings was due to a significant rank change in RC root yield between 2006 and 2007. RC had much lower root yield in 2006 than 2007 while JR, PT and SQ roots had similar yields in both 2006 and 2007 (data not shown). Despite the C x Y interaction, JR had the highest root yield (14 $Mg \cdot ha^{-1}$) while SQ had the lowest root yield (11 $Mg \cdot ha^{-1}$) in the May planting for both years of the study. Mulch had a significant effect on yield with silver and yellow mulch treatments resulting in the highest root yield (13 $Mg \cdot ha^{-1}$) and no mulch plots resulting in the lowest root yield (11 $Mg \cdot ha^{-1}$) in May plantings. In August plantings only C, Y and the C x Y interaction were significant factors influencing root yield (Table 2.4). The C x Y interaction was due to rank changes between all the cultivars between the two years of the study. In 2006, PT

yielded the most root biomass (20 $Mg \cdot ha^{-1}$) and SQ the least (12 $Mg \cdot ha^{-1}$), while in 2007 JR yielded the most root biomass (13 $Mg \cdot ha^{-1}$) while RC (8 $Mg \cdot ha^{-1}$) yielded the least.

Glucosinolate concentration. GSL profiles differed between shoot and root tissues. Within a cultivar, shoots had slightly less total GSLs (TTL; Table 2.5) than roots (Table 2.6), except for JR where shoots had roughly the same amount of TTL as roots. This is in agreement with previous research which has found GSL concentrations to be higher in root tissues than shoot tissues (Carlson et al., 1987; van Dam et al., 2009). Root profiles also differed from shoot profiles in that roots contained higher proportions of the aromatic GSL gluconasturtiin (GNS; 2-phenyethyl GSL) and the indolic GSL 1-methoxyglucobrassicin (1MGB; 1-methoxy-3-ylmethyl GSL) than shoot tissues (Table 2.6). These tissue-dependent differences in GSL profiles are consistent with previous data showing that root tissues contain a more diverse GSL profile than shoot tissues (as reviewed by van Dam et al., 2009). The diversity of GSL profiles in root tissues may have evolved to combat constant pathogen pressures in soils (van Dam et al., 2009).

Shoot GSL profiles differed by cultivar with the aliphatic GSLs gluconapin (GNP; 3-butenyl GSL), glucobrassicanapin (GBN; 4-pentenyl GSL), gluconapoleiferin (GF; 2-hydroxy-4-pentenyl GSL) and progoitrin (PRO; 2-hydroxy-3-butenyl GSL) contributing to over 90% of the profile on average across all cultivars and planting dates (Table 2.5). GNP and GBN were the primary GSLs in JR shoots across both planting dates, comprising on average 64% and 32% of the GSL profile, respectively. Our results are consistent with Carlson et al. (1987) who also found GNP and GBN to be the predominant GSLs in JR shoot tissue, comprising 55% and 31% of total GSLs. GBN and GNP were also the primary GSLs in SQ shoots across both planting dates, comprising on average 48% and 36% of the profile, respectively. The high concentration of GNP found in JR and SQ shoot tissues is also consistent with previous research citing GNP as the predominant shoot GSL in turnip shoot tissue (Smetanska et al., 2007). RC and PT shoots had similar profiles to each other in both planting dates with GBN and PRO being the primary GSLs comprising 44% and 20% of the profile, respectively. These results agree

with Carlson et al. (1987) who found that in RC and PT, GBN comprised 47% of the GSL profile.

Planting date X year interactions were significant for many GSLs, therefore data for root and shoot GSL concentrations are presented individually by planting date (Tables 2.5 and 2.6). In May plantings, JR shoots had the highest TTL (164 μmol·100g^{-1} fw) of the four cultivars examined (Table 2.5). The C x Y interaction was significant for GNP, however, despite the interaction, JR consistently had the highest GNP concentration (101 μmol·100g^{-1} fw) in both years of the study. The C x Y interaction was also significant for GF, however, despite the significance of the interaction, PT and RC shoots consistently had the highest GF (17 and 15 μmol·100g^{-1} fw, respectively). GBN was consistent across years of study with RC shoots having lower GBN than the other cultivars in May plantings. The C x M interaction for PRO in May planted shoots was significant, however despite this interaction, PT and RC shoots had the highest PRO concentrations (24 μmol·100g^{-1} fw) across all mulch treatments.

In August plantings, the C x M x Y interaction was significant for TTL and GBN in shoot tissues (Table 2.5). The 3-way interaction for TTL resulted from changes in rank across cultivars, mulch treatments and years of the study, with mulches only consistently significantly influencing TTL in SQ shoots in both years of the study (Table 2.8). The 3-way interaction for GBN also resulted from changes in rank between cultivars depending on the mulch treatment and year of the study (data not shown). The C x Y interaction was significant for all individual GSLs in the August planting. Despite the significance of the C x Y interaction, JR shoots consistently had the highest GNP concentration (92 μmol·100g^{-1} fw) and PT and RC had the highest PRO and GF concentrations (on average 31 and 15 μmol·100g^{-1} fw for PRO and GF, respectively) in both years of the study. These cultivar differences in GSL concentrations are similar to those observed in May planted turnip shoots (Table 2.5).

Root GSL profiles differed by cultivar (Table 2.6). In JR roots, GNP and GNS were the primary GSLs comprising 42% and 33% of the GSL profile, respectively. RC and PT roots had similar profiles with GNS and PRO comprising on average 28% and

25% of the profile, respectively. In SQ roots, GNS and GBN were the primary GSLs comprising 27% and 24% of the profile respectively. 1MGB was the most abundant indolic GSL in turnip tissues. In SQ roots 1MGB comprised 7% (16 µmol·100g^{-1} fw) of the profile on average. In all other cultivars and tissues 1MGB comprised less than 3% (4µmol·100g^{-1} fw) of the profile. Our results are consistent with previous research reporting GNS to be high in root tissues of *Brassica rapa* (Zhang et al., 2008) and other members of the order Brassicales (as reviewed by van Dam et al., 2009). However, our results are inconsistent with other research showing 1MGB to be the primary root GSL in teltower turnip (Brassica *rapa var*. *rapa pygmaea teltoviensis*; Smetanska et al., 2007). This discrepancy may be due to inherent cultivar-dependent differences in GSL profiles.

The C x M x Y interaction was significant for TTL, GF, GNP and GNS in May planted roots and significant for GF, GNP and GBN for August planted roots (Table 2.6). The C x Y interaction was significant for all individual root GSLs for both planting dates (Table 2.6). The 3-way interaction for TTL in May planted roots resulted from changes in rank of across cultivars, mulch treatments and years of the study (Table 2.9). In many instances SQ roots had significantly higher TTL than JR however this trend was not significant across all mulch treatments in May plantings in both years of the study. In August plantings the C x Y interaction was significant for TTL (Table 2.6). The significance of the C x Y interaction was mainly due to changes in rank between RC and PT between 2006 and 2007. Despite the C x Y interaction, SQ roots had the highest TTL in August plantings while JR had the lowest TTL (252 and 141µmol·100g^{-1} fw for SQ and JR, respectively). PT roots had intermediate TTL between SQ and JR in August plantings with average TTL of 198 µmol·100g^{-1} fw. This concentration is similar to those observed by Antonious et al. (1996) who reported a range of total GSL concentrations of approximately 160 to 275 µmol ·100g^{-1} fw in PT roots depending on the mulch treatment.

Despite the C x M x Y and C x Y interactions present for individual root GSLs at both planting dates, some cultivar-dependent trends existed in this study. JR always had the lowest PRO (~ 9 µmol·100g^{-1} fw) and the highest GNP (~ 62 µmol·100g^{-1} fw) across years and planting dates. These values are similar to those observed in JR shoots. SQ

always had the highest GBN (43 and 72 µmol·100g^{-1}) and 1MGB (25 and 7 µmol·100g^{-1} fw) for May and August plantings, respectively.

It is possible that cultivar differences in root GSL concentrations could have been due to a dilution effect as JR roots have lower GSL concentrations than SQ roots, but larger root yields than SQ. We attempted to compensate for dilution effects by using roots of comparable size (5-7.6 cm diameter) for GSL analysis. When root GSL concentrations are adjusted based on yield, JR roots still have lower GSL content (2130 mol TTL·ha^{-1}) than SQ roots (2530 mol TTL·ha^{-1}) suggesting these cultivar differences in GSL concentrations are not due to a dilution effect.

The influence of mulch on GSLs. Although colored plastic mulches significantly influenced both total and individual GSL concentrations, mulch-dependent increases in GSL concentrations were not consistent across tissue types, cultivars, planting dates and years of the study. In the May planting, M x Y interactions were significant for TTL and all individual shoot GSLs (Table 2.5). The M x Y interaction resulted from red and blue mulches yielding significantly more TTL (186 and 177 µmol·100g^{-1}fw, respectively) than the other mulches in 2006 but not 2007 (Figure 2.1). Despite the significance of the M x Y interaction, blue mulches yielded the highest GNP in both years of the study in May plantings. In August plantings, the C x M x Y interaction was significant for TTL and GBN in shoots (Table 2.5). This 3-way interaction was due to cultivar-dependent differences in the effect of mulch treatments on TTL (Figure 2.2) and GBN (data not shown) between the two years of the study. Mulch was not a significant factor for any other GSLs in August planted shoots.

In May planted roots, the C x M x Y interaction was significant for TTL, GF, GNP and GNS (Table 2.6). This interaction was due to differences in the effect of mulches on TTL and individual GSLs in turnip cultivars between the two years of the study. The C x M x Y interaction for TTL is depicted in Figure 2.3. C x M interactions were significant for GF, GNP and GBN. In all cases, the C x M interactions in May planted roots were due to rank changes between cultivars and mulches for GSL concentrations. M x Y interactions were significant for all other individual GSLs except

PRO. The M x Y interaction present for most GSLs in May planted roots was primarily due to significant differences between mulches being present for individual GSLs in 2006 but not 2007. Mulch was significant without any interactions for PRO, with blue mulch yielding on average 30% more PRO than the other mulch treatments (Table 2.6).
In August plantings, mulch was significant for TTL and 1MGB in roots (Table 2.6). No mulch plots yielded the highest TTL in roots (198 μmol·100g^{-1} fw) while blue and yellow mulches consistently yielded the lowest TTL across cultivars (179 and 180 μmol·100g^{-1} fw, respectively). No mulch control plots also yielded the highest root 1MGB concentration (4 μmol·100g^{-1} fw) across cultivars. M x Y interactions were significant for GF, GNP and GNS due to rank changes in the mulch treatments between the two years of the study.

The relationship between GSLs, mulch and climatic properties. In our study, mulch treatments altered both soil temperatures (Table 2.2) and reflected light properties (Table 2.1). Mulches reflected more light than the soil surface (Table 2.1; no mulch) with white mulch reflecting the most photosynthetic photon flux (PPF). This is similar to what Antonious et al. (1996) observed. Blue mulch reflected the most blue light and the least red (R) and far-red (Fr) light. Red mulch reflected the most red and far-red light. Silver, white and yellow mulches reflected higher amounts of green light than blue and red mulches. Although our mulch treatments varied in terms of their reflected light properties, we found no consistent relationship between the spectral properties of our mulch treatments and GSL concentrations, particularly GSL concentrations in root tissues. For example, $r=0.02$, $p>0.05$ for reflected blue light and TTL. This is in contrast to results reported by Antonious et al. (1996) who attributed the increased GSL concentrations in their mulch treatments to the amount of blue light the mulches reflected. This contradiction between the two studies may be due to differences in planting density and exposed mulch surface. Our trial utilized a spacing of 5cm between plants, while Antonious et al. (1996) utilized a spacing of 30cm between plants. It is possible that the lower plant density in their study than in our study resulted in different reflectance properties by the mulch into the crop canopy.

Antonious et al. (1996) suggested that changes in total GSL concentrations observed in turnip roots grown with colored plastic mulches were mainly due to changes in reflected light provided by mulches rather than soil temperature as the range of soil temperatures between the mulch treatments in their study was less than 2°C. In our study the range between mean soil temperatures of all mulch treatments within each planting date was approximately 8°C (Table 2.2). Blue and red mulches had the highest mean soil temperatures in most planting dates of the study (Table 2.2). In May plantings, blue and red mulches were 4-6°C warmer than no mulch treatments. This soil temperature difference was mitigated in August plantings where soil temperatures under blue and red mulch treatments were only 1-4°C warmer than no mulch treatments. Indeed there was a slight positive, but significant correlation between soil temperature and TTL in roots ($r=0.17$, $p<0.001$). The positive relationship between soil temperature and TTL we observed is in agreement with previous research where elevated temperatures are associated with increases in GSL concentrations (Charron and Sams, 2004; Charron and Sams, 2005).

Although we did not observe consistent differences in GSL concentrations between our mulch treatments, we did observe large differences in GSL concentrations between planting dates and years of our study. Seasonal differences in GSL accumulation have been positively associated with mean air temperatures and solar radiation, particularly within the two weeks prior to harvest (Charron and Sams, 2005). In our study, mean air temperatures, growing degree days and solar radiation levels were higher in May plantings than August plantings, particularly during the 10 days prior to harvest. The highest mean GSL concentrations we observed were in the August 2006 planting (Table 2.5 and Table 2.6) which had lower mean air temperatures and solar radiation levels than the May planting, particularly near harvest (Table 2.3). Indeed, a small, but significant negative correlation existed between mean maximum air temperature and shoot TTL ($r=-0.15$ and $r=-0.21$, $p<0.001$, for harvest and growing season, respectively). Thus, our results do not agree with those of Charron and colleagues

(2005) who observed a positive relationship between GSL concentration and temperature and GSL concentration and photoperiod, particularly right before harvest.

2.5 Conclusions

The presence of significant interactive effects between cultivars, plastic mulch treatments and years in influencing GSL concentrations across planting dates suggests that mulch treatments alter GSL levels in a cultivar and climate dependent manner. Thus using colored plastic mulches to increase the phytonutrient properties of turnips may not give consistent results. The inconsistency of mulch-dependent GSL enhancement may be due to interactions between temperature, solar radiation and mulch properties as these factors individually had a small but significant influence on GSL concentrations. Because of these interactions, we conclude that colored plastic mulches do not provide a sufficient effect to overcome the influence of climatic factors on GSL concentrations in turnip roots and shoots.

Table 2.1 Quantities of reflected light measured 10 cm above different colored mulches or soil surface (no mulch).

	Reflected light ($\mu mol \cdot m^{-2} \cdot s^{-1}$)					
	Mulch Surface Color					
Light Characteristic	Blue	Red	Silver	Yellow	White	No Mulch
PPF (400-700 nm)	22	33	31	27	46	2
Blue (445-455 nm)	54	7	36	4	51	1
Green (545-555 nm)	9	6	32	42	49	2
Red (R; 640-650 nm)	7	49	28	39	45	3
Far-red (Fr; 735-745 nm)	13	68	29	34	41	3
R:Fr ratio (ratio of 660:740)	0.98	0.94	1.08	1.17	1.16	0.92

Table 2.2 Mean soil temperatures 5cm below soil surface under colored mulch treatments for each planting date and year.

		Mean seasonal soil temperatures (ºC)					
		Mulch Surface Color					
Planting	Year	Blue	Red	Silver	Yellow	White	No Mulch
May	2006	27.5	26.6	21.9	22.3	20.8	22.4
	2007	27.0	26.7	19.3	21.4	22.0	20.7
August	2006	23.0	21.0	21.3	20.3	19.2	18.9
	2007	19.4	19.8	14.2	16.0	16.7	18.3

Table 2.3 Climatic data for each growing season and ten days prior to harvest for each planting date and year.

Planting	Year	Mean Daily Maximum Air Temperature (°C)		Mean Daily Minimum Air Temperature (°C)		Cumulative Growing Degree Units		Mean Daily Solar Radiation (Langleys)	
		Season	Harvest	Season	Harvest	Season	Harvest	Season	Harvest
May	2006	24	27	13	14	813	188	512	534
	2007	26	28	13	17	866	213	534	584
August	2006	23	19	13	9	760	99	358	319
	2007	24	22	11	11	632	144	306	153

Table 2.4 Fresh weight yield of turnip shoots and roots in May and August planting dates across years, cultivars and plastic mulch treatments.

	Fresh Weight ($Mg \cdot ha^{-1}$)			
	SHOOTS		ROOTS	
Source of Variation[1]	May	August	May	August
Cultivar (C)				
JR	23±1	24±1	14±0	15±0
PT	17±0	17±1	13±0	14±1
RC	20±1	17±1	12±0	12±1
SQ	14±0	14±0	11±0	11±0
Significance	*[z]	*[z]	*[z]	*[z]
Mulch (M)				
None	18±1 ab	21±1 a	11±0 b	12±1
Blue	18±1 ab	18±1 b	12±0 a	13±1
Red	17±1 b	18±1 b	13±0 a	13±1
Silver	19±1 a	17±1 b	13±0 a	13±1
White	19±1 ab	17±1 b	13±1 a	14±1
Yellow	19±1 a	17±1 b	12±0 a	13±1
Significance	*	*	*	NS
Year (Y)				
2006	19±1	20±1	13±0	16±1
2007	17±1	16±1	12±0	10±0
Significance	*[z]	*[z]	*[z]	*[z]
Interactions				
C×M	NS	NS	NS	NS
C×Y	*	*	*	*
M×Y	NS	NS	NS	NS
C ×M ×Y	NS	NS	NS	NS

[1]Means with different letters within each column and source of variation are significantly different at $P \leq 0.05$ as determined by Tukey's HSD.
Significance:[NS,*] Nonsignificant or significant at $P \leq 0.05$.

[z]Mean separations on main effects were not performed due to the presence of significant interactions.

Table 2.5 Glucosinolate concentrations of turnip shoots in May and August planting dates across cultivars, plastic mulch treatments and years.

Source of Variation[1]	Glucosinolate Concentration (μmol·100g^{-1} fw)									
	PRO		GF		GNP		GBN		TTL	
	May	August	May	August	May	August	May	August	May	August
Cultivar (C)										
Just Right	1±0	1±0	1±0	1±0	101±5	92±5	54±4a	41±2	164±8a	138±6
Purple Top	24±2	31±2	17±1	16±1	18±2	23±1	56±3a	84±4	131±8b	167±7
Royal Crown	24±2	31±2	15±1	14±1	17±2	16±1	36±2b	68±3	114±6b	145±5
Scarlet Queen	6±1	6±1	10±1	7±1	36±3	62±3	57±4a	74±4	117±8b	158±7
Significance	*[z]	*[z]	*[z]	*[z]	*[z]	*[z]	*	*[z]	*	*[z]
Mulch (M)										
None	15±2	20±3	12±2	9±1	43±5	43±6	45±4	53±5	127±9	137±8
Blue	17±3	18±3	12±2	10±2	58±8	54±8	59±4	76±4	158±10	165±7
Red	18±3	19±3	14±2	10±1	46±7	47±6	63±5	72±6	156±10	158±9
Silver	9±2	17±3	8±1	10±1	35±7	45±6	45±4	63±4	107±10	144±6
White	13±3	16±3	11±2	10±1	41±8	51±7	49±4	72±4	125±10	157±7
Yellow	10±2	14±2	7±1	8±1	36±6	51±6	44±3	66±6	114±7	151±8
Significance	*[z]	NS	*[z]	NS	*[z]	NS	*[z]	*[z]	*[z]	*[z]
Year (Y)										
2006	14±2	17±2	14±1	10±1	41±4	58±5	53±3	71±3	138±7	167±5
2007	13±1	18±2	7±1	9±1	46±4	39±2	49±2	62±3	124±5	136±4
Significance	NS	NS	*[z]	NS	NS	*[z]	NS	*[z]	*[z]	*[z]
Interactions										
C×M	*	NS	NS	NS	NS	NS	NS	*	NS	NS
C×Y	NS	*	*	*	*	*	NS	*	NS	*
M×Y	*	*	*	NS	*	NS	*	*	*	*
C ×M ×Y	NS	NS	NS	NS	NS	NS	NS	*	NS	*

Table 2.6 Glucosinolate concentrations of turnip roots in May and August planting dates across cultivars, plastic mulch treatments and years.

Source of Variation[1]	Glucosinolate Concentration (μmol·100g^{-1} fw)													
	PRO		GF		GNP		GBN		1MGB		GNS		TTL	
	May	August	May	August	May	August	May	August	May	August	May	August	May	August
Cultivar (C)														
Just Right	11±1	6±1	2±0	0±0	63±3	62±2	23±1	21±0	7±2	2±2	47±1	49±0	154±5	141±4
Purple Top	49±3	47±1	25±1	21±1	18±2	23±3	20±1	55±2	5±2	1±3	53±0	42±0	182±5	198±6
Royal Crown	46±2	40±1	24±2	11±1	10±1	21±2	20±1	36±1	6±2	2±3	65±1	47±0	185±6	162±6
Scarlet Queen	49±2	43±3	23±3	22±3	12±1	27±2	43±2	72±1	25±2	7±3	57±1	71±0	217±7	252±9
Significance	*[z]	*[z]	*[z]	*[z]	*[z]	*[z]	*[z]	*[z]	*[z]	*[z]	*[z]	*[z]	*[z]	*[z]
Mulch (M)														
None	41±4b	34±4	23±3	14±2	24±4	37±4	28±2	48±4	13±2	4±4a	51±2	55±1	190±8	198±12a
Blue	48±4a	34±4	19±3	10±2	30±5	29±4	29±2	45±4	12±2	3±4ab	58±2	55±0	206±7	180±10b
Red	34±4b	34±4	17±3	15±2	30±6	33±4	25±2	45±4	10±3	3±3ab	47±1	52±0	171±6	188±10ab
Silver	37±3b	36±4	22±3	14±3	24±4	33±4	26±2	47±4	10±3	2±3b	61±1	51±0	189±9	190±10ab
White	35±4b	33±4	16±2	15±3	22±4	37±4	25±3	47±4	9±3	2±4ab	57±2	53±0	171±10	194±13ab
Yellow	38±4b	32±4	15±2	14±3	25±4	32±4	26±2	43±4	9±2	3±3ab	61±1	49±1	181±6	179±11b
Significance	*	NS	*[z]	*[z]	*[z]	*[z]	NS	NS	*[z]	*	*[z]	*[z]	*[z]	*
Year (Y)														
2006	32±2	43±2	25±2	20±2	25±2	45±2	23±1	44±2	13±2	3±2	57±1	67±0	185±5	226±6
2007	46±2	25±2	12±1	8±1	27±3	23±2	30±2	48±2	8±1	2±1	54±1	38±0	184±4	149±4
Significance	*[z]	*[z]	*[z]	*[z]	*[z]	*[z]	*[z]	*[z]	*[z]	*[z]	NS	*[z]	NS	*[z]
Interactions														
C×M	NS	NS	*	*	*	*	*	NS	NS	NS	NS	NS	NS	NS
C×Y	*	*	*	*	*	*	*	*	*	*	*	*	*	*
M×Y	NS	NS	*	*	*	*	*	NS	*	NS	*	*	*	NS
C ×M ×Y	NS	NS	*	*	*	*	NS	*	NS	NS	*	NS	*	NS

[1]Means with different letters within each column and source of variation are significantly different at $P \leq 0.05$ as determined by Tukey's HSD. Progoitrin (PRO); Gluconapoleiferin (GF); Gluconapin (GNP); Glucobrassicanapin (GBN); 1-methoxyglucobrassicin (1MGB); Gluconasturtiin (GNS); Total (TTL).
Significance:[NS,*] Nonsignificant or significant at $P \leq 0.05$.

Figure 2.1 The interactive effects of colored plastic mulch and year on total glucosinolate concentrations in May planted shoots. Bars with different letters within each year are significantly different at P≤0.05 as determined by Tukey's HSD.

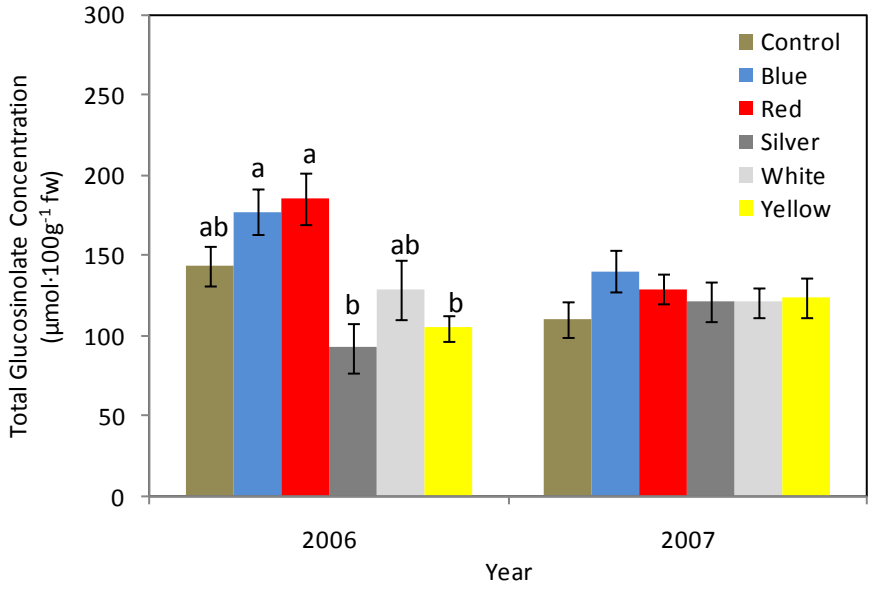


Figure 2.2 The interactive effects of colored plastic mulch, cultivar and year on total glucosinolate concentrations in turnip shoots grown in August plantings. Bars with different letters within each cultivar and year are significantly different at P≤0.05 as determined by Tukey's HSD. (A)Just right (B) Purple Top (C) Royal Crown (D) Scarlet Queen.

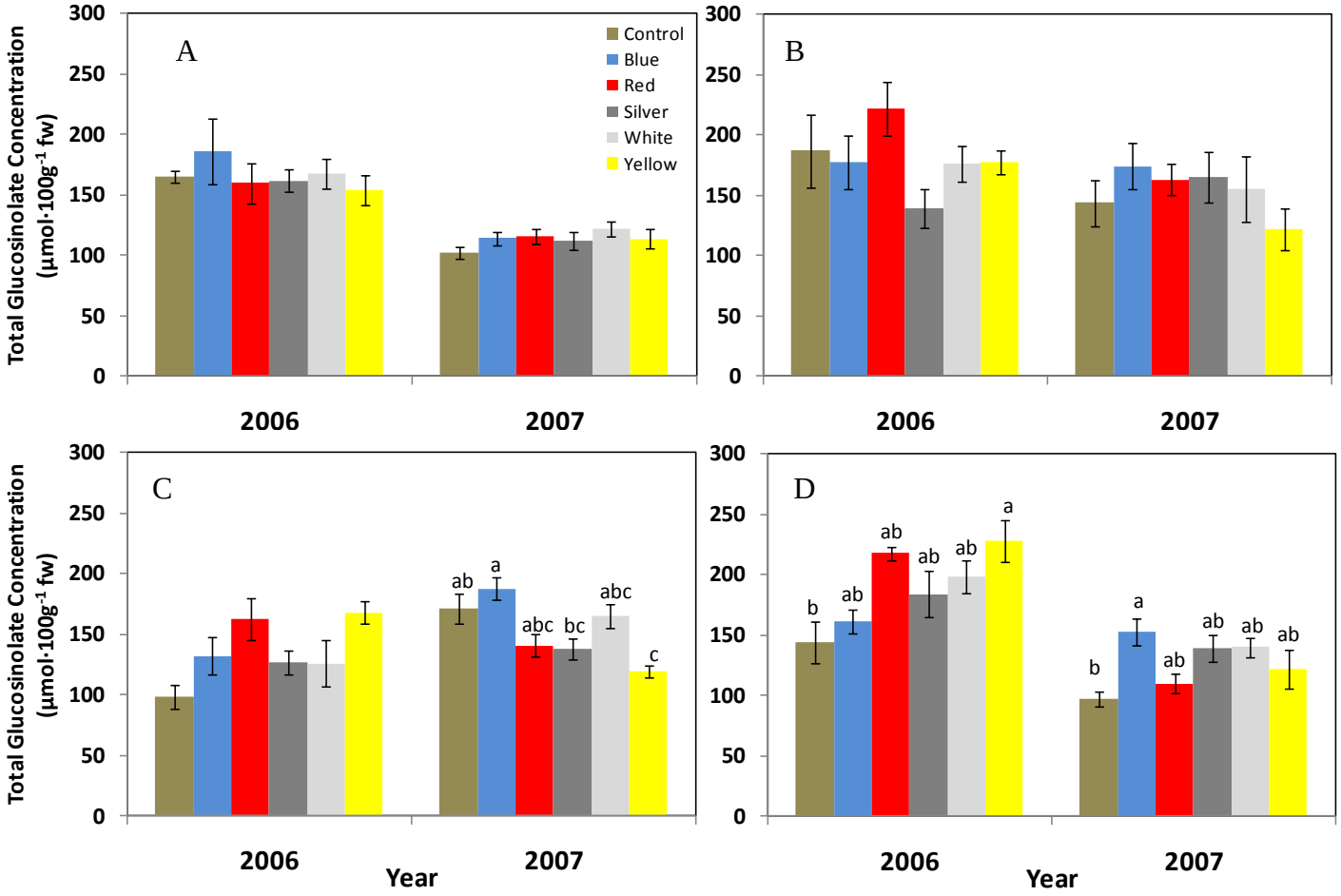
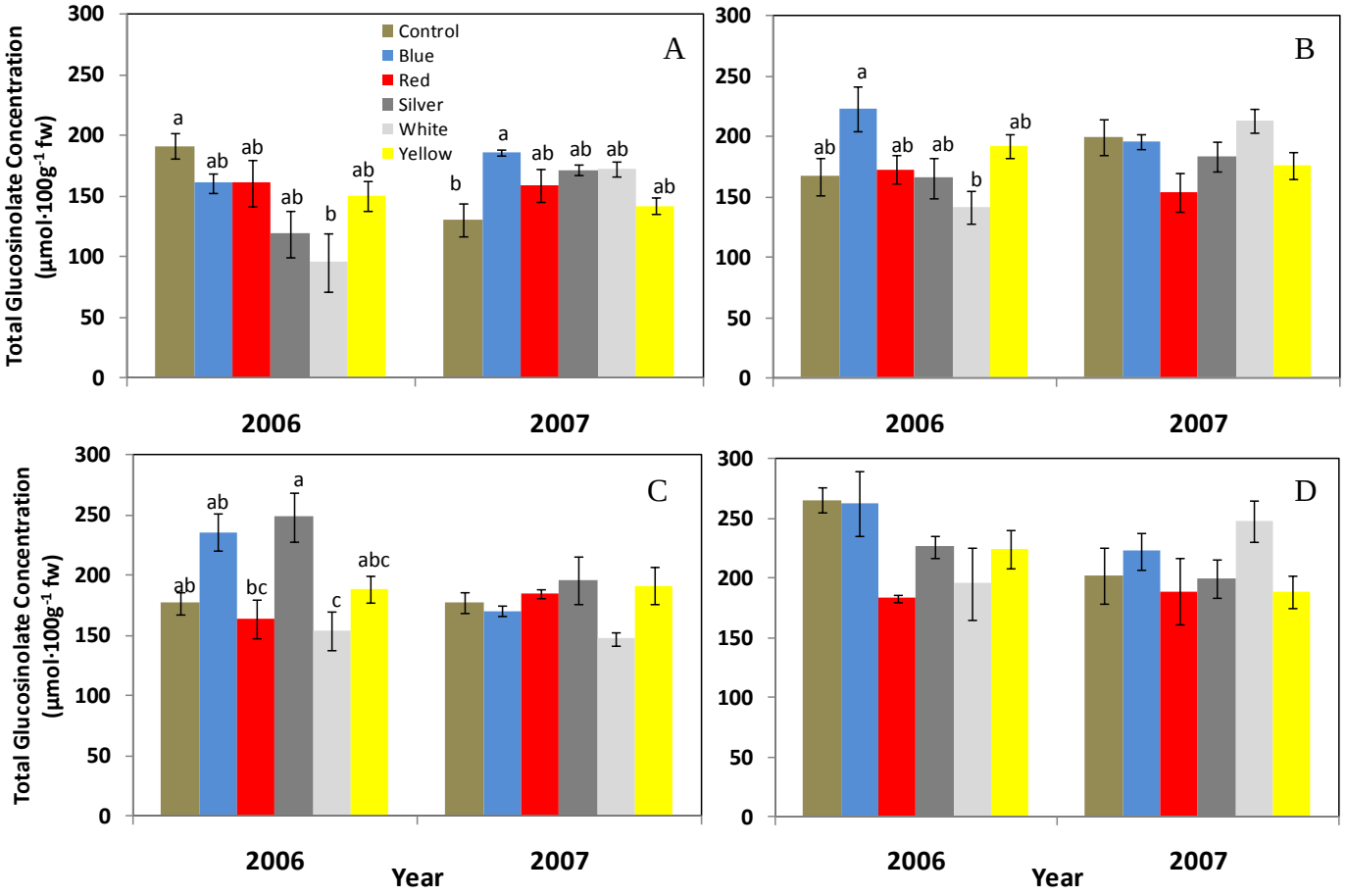


Figure 2.3 The interactive effects of colored plastic mulch, cultivar and year on total glucosinolate concentrations in turnip roots grown in May plantings. Bars with different letters within each cultivar and year are significantly different at P≤0.05 as determined by Tukey's HSD. (A)Just right (B) Purple Top (C) Royal Crown (D) Scarlet Queen.



3 Seasonal variation in glucosinolate accumulation in turnips grown under photoselective nettings

3.1 Summary

Glucosinolates (GSLs) are secondary metabolites produced by cruciferous vegetables whose hydrolysis by-products have significant value for plant defense and human health. GSL accumulation is greatly influenced by environmental conditions including planting date and light quality and quantity. Photoselective nettings influence both the quality and quantity of light transmitted onto the crop canopy. The objective of this study was to determine yield and GSL concentrations in turnip roots and shoots grown under different photoselective nettings at different planting dates. Field studies were conducted with red ('Scarlet Queen'; SQ) and white ('Just Right'; JR) turnip cultivars at May and August planting dates for two years using blue, red and yellow photoselective nettings and a no netting control. Netting did not significantly affect shoot yield and had an inconsistent effect on root yield. Cultivars differed in GSL concentrations with JR having the highest gluconapin (3-butenyl GSL) concentrations in root and shoot tissues and SQ having the highest total GSL concentration in root tissues. Netting did not significantly influence total or individual GSLs in root tissues. Netting was only a significant factor for glucobrassicanapin (GBN; 4-pentenyl GSL) concentration in shoots with no netting treatments resulting in the highest GBN concentrations. May plantings resulted in 50% higher total GSL concentrations than August plantings. Planting date x year interactions were significant for GSL concentrations and proportions. These interactions may be due to differences in mean air temperatures and solar radiation prior to harvest.

3.2 Introduction

Glucosinolates (GSLs) are nitrogen and sulfur-containing secondary metabolites characteristic of plants in the order Brassicales. The general structure of GSLs is a glucose bound to a modified amino acid via a thioester bond. Three groups of GSLs exist: aromatic GSLs derived from phenylalanine or tyrosine; indolic GSLs derived from tryptophan; and aliphatic GSLs primarily derived from methionine. GSLs react with a

thioglucosidase, myrosinase, that hydrolyzes the thioester bond and creates by-products such as isothiocyanates. These hydrolysis by-products have significant values for human health (Hecht, 2000; Neave et al., 2005), plant defense (i.e. Mewis et al., 2006), and soil biofumigation (Gimsing and Kirkegaard, 2009).

GSL accumulation is highly dependent on environmental conditions such as soil fertility (Kim et al., 2001; Rosen et al., 2005), planting date (Charron and Sams, 2004; Radovich et al., 2005; Zhang et al., 2008), light quality (Antonious et al., 1996; Engelen-Eigles et al., 2006) and light quantity (Charron and Sams, 2004). Defining and manipulating cultural practices to maximize GSL concentration in vegetables could have significant impacts on human health and the nutraceutical industry.

Several studies suggest that light quality can influence GSL concentration. Antonious and colleagues (1996) found that turnips grown with different colored mulches (black painted blue, white or green) resulted in differential increases in total GSLs and sugars. Justen and Fritz (Chapter 2) also found that total GSL levels as well as individual GSLs in turnip roots and shoots can be influenced by different colored mulch treatments. Engelen-Eigles et al. (2006) concluded that a continuous red light treatment in a growth chamber over a two week period increased the aromatic GSL gluconasturtiin by 39% in watercress when compared to metal halide and far-red light treatments. Lefsrud et al. (2008) found that specific wavelengths of LED irradiance increased the sinigrin (SN) concentration in kale leaves. This boost in SN may not be solely due to wavelength of light, as the 640 nm LED array which resulted in the highest SN concentrations also had the highest irradiance, approximately 10-fold higher than the other arrays tested.

Expression of genes involved in GSL biosynthesis can also be influenced by light quality. Hoecker et al. (2004) found that continuous red light, but not continuous far-red light increased CYP83B1 transcript levels in *A. thaliana* seedlings. The CYP83B1 gene codes for a cytochrome P_{450} enzyme responsible for the first committed step in indolic GSL biosynthesis (Bak et al., 2001). Therefore light–induced changes in gene expression could increase indolic GSL concentration. Expression of other GSL biosynthesis and regulatory genes can influence the concentration of individual and total GSLs (Grubb and

Abel, 2006). It also has been suggested that GSL regulatory genes such as MYB transcription factors greatly influence GSL concentrations in response to environmental conditions and may function in a tissue-specific manner (Hirai et al., 2007). However, the effect of light quality on the differential expression of these genes in different tissues has not been confirmed.

One way to alter the light quality in a field setting is by using photoselective (colored shade) nettings. These nets have the ability to influence the amount and spectra of radiation transmitted to crops, but also can influence environmental conditions such as relative humidity, temperature and air movement (as reviewed by Stamps, 2009). Photoselective nettings have been used in many crops to alter vegetative growth, flowering and crop yield and quality. Photoselective nettings have mostly been used with floral, ornamental foliage (Stamps, 2008) and fruit crops (Retamales et al., 2008), however, some research has been done to assess the use of netting in bell pepper (*Capsicum annuum*) production (Elad et al., 2007; Fallik et al., 2008). Elad and colleagues (2007) found that nettings increased yield and fruit quality in bell peppers when compared to a no netting control. No information on the use of photoselective nettings to alter secondary metabolites such as GSLs is available.

GSL concentrations and profiles vary between tissue, cultivar and species (van Dam et al., 2009). Turnip (*Brassica rapa*) is a unique species as it accumulates edible biomass both above and below ground and has a short generation time, allowing for multiple plantings in a growing season. Additionally, turnip has distinct GSL profiles in shoot and root tissues, with aliphatic GSLs predominating shoot tissues and aromatic and indolic GSLs predominating root tissues (Carlson et al., 1987; Smetanska et al., 2007). Because GSL biosynthesis can be influenced by light quality and may be dependent on the type of GSL and the tissue the GSL accumulates in, turnips provide an interesting model to explore the effect of environmental manipulation on whole-plant GSL profiles. The objective of this study was to quantify yield and GSL concentrations in turnip roots and shoots grown under different photoselective nettings in both fall and spring production seasons.

3.1 Materials and Methods

'Just Right' (JR; Jordan's Seed, Woodbury,MN) and 'Scarlet Queen' (SQ; Johnny's Seeds, Winslow, ME) turnips were hand seeded in raised beds (1.5 m apart on center, 2.1 m long) at 5 cm plant spacing. These cultivars were chosen based on their GSL profiles as observed in the plastic mulch experiment (Chapter 2). In this study JR roots generally had the lowest total GSLs in root tissues while SQ had the highest total GSLs in root tissues of the cultivars tested. Netting treatments included red, yellow and blue photoselective netting and no netting control (40% shade ChromatiNet; Polysack USA, Inc., San Diego, CA). The spectral properties of light transmitted through the netting treatments are shown in Table 3.1 and Figure S6. Light quantities transmitted through the photoselective nettings were measured using an Apogee Model Spec-UV/PAR spectroradiometer (Apogee Instruments, Inc., Logan, UT). Trasmitted R: FR ratios were measured with a Skye red-far red meter (Skye Instruments, Ltd., Powys, UK).

The study was conducted at the Southern Research and Outreach Center in Waseca, Minn. (44.07° N , 93.52°W) on a Webster Clay loam soil (fine-loamy, mixed superactive typic endoaquoll with a pH of 6.5 and soil P and K of 45 ppm and 159 ppm, respectively) soil. Plots were arranged in a Randomized Complete Block Design with each netting and cultivar combination being randomized within each of three replicates. Planting dates were 13 May and 4 August 2008 and 12 May and 5 August 2009. Netting was applied over emerged seedlings on 21 May and 11 August 2008 and 20 May and 11 August 2009 using 80 cm tall by 80 cm wide frames as depicted in Figure S7. To gauge the effect of netting treatments on temperature, soil temperatures under each netting type were monitored 5cm below the soil surface for all planting dates using an Optic StowAway® data logger, model WTA08 (Onset Computer Corp., Pocasset, MA; Table 3.2; Figure S8). Seasonal temperature and solar radiation data were obtained from the Southern Research and Outreach Center weather information website (Table 3.3; http://sroc.cfans.umn.edu/ WeatherInformation/index.htm).

Plots were fertilized with 67kg·ha^{-1} N pre-plant and irrigated as needed with drip irrigation to provide a minimum of 2.5 cm of water each week. Conventional

management practices were utilized for pest and weed management. Ten plants were harvested from each plot on 30 June and 19 September 2008 and 29 June and 22 September 2009. Plants were harvested between 09:00-11:00h. Plants were topped, and shoot and root weights were taken separately for yield measurements. Only roots 5-7.6 cm in diameter were used for GSL analysis. All samples were stored at 4°C prior to processing for GSL quantification and were processed within five days after harvest.

Glucosinolate quantification. Extraction and GSL quantification was performed as per Hect et al. (2004), utilizing modifications from Rosen et al. (2005). Briefly, a 100 g leaf sample or 150 g root sample was boiled in 700 mL of boiling water for 5 min to deactivate myrosinase. Boiled samples were macerated in a blender for 2 min. A 40 mL-aliquot of blended sample was homogenized using a BioSpec M133 Homogenizer (BioSpec Products, Inc., Bartlesville, OK) set at 12,000 rpm for 2 min, then centrifuged for 10 min at 5000 *gn*, 4°C.

Desulfoglucosinolate (ds-GSL) extraction was performed using conditioned solid phase strong anion exchange (SAX) columns (Sigma-Aldrich, St. Louis, MO). Sinigrin (2-propenyl GSL; Sigma-Aldrich, St. Louis, MO) was added to the conditioned SAX columns as an internal standard. To desulfate, samples were incubated with two units (0.2mg/mL) of sulfatase (aryl-sulfate sulfohydrolase; EC 3.1.6.1; Sigma-Aldrich, St. Louis, MO) on SAX columns for ~15 hr at room temperature (~21°C) then eluted with 3mL water and the collected volume was determined by weight. Further washing of the columns yielded no additonal ds-GSLs confirming complete elution. Eluent was stored at -20°C until HPLC analysis.

HPLC analysis was performed on an Agilent 1200 Series Quaternary system (Agilent technologies, inc., Santa Clara, CA) set at λ=229 nm using a Luna C18, 5µm, 250 x 4.6 mm column (Phenomenex, Torrance, CA) set at 30ºC. A 50-µL aliquot of the eluent was separated on the system with a flow rate of 1.0mL·min^{-1} using the following gradient: solvent A= water and B= acetonitrile; 0 to 2 min, 95% A, 5%B; 2 to 20 min, 85% A, 15% B; 20 to 23min, 53% A, 47% B; 23 to 30 min, 0% A, 100% B; and 30 to 33 min, 95% A, 5% B. Peaks were integrated using ChemStation for LC 3D Systems, Rev.

B.04.01 software. A sample chromatogram is presented in Figure S4. GSL peak identities were confirmed using retention time and Ultra Performance Liquid Chromatography-Mass Spectrometry (Waters Corporation, Milford, MA) using a C18 column, a water:acetontirile gradient and negative electrospray ionization (see Figure S5 for an example mass spectra). Ds-GSL concentrations were calculated using relative quantification with an internal standard (sinigrin) and previously published response factors (EU, 1990). Ds-GSL concentrations are reported on a $\mu mol \cdot 100g^{-1}$ fw basis.

Statistical Analysis. Data were analyzed with R 2.9.1 (R Foundation for Statistical Computing, Vienna, Austria). The significance of differences between treatments, cultivar and experiments was assessed by a fixed-factor ANOVA. Data are expressed as means. Mean values were considered significantly different at $P < 0.05$ as determined by Tukey's HSD.

3.2 Results and Discussion

Yield. Shoot and root yield were significantly affected by Cultivar (C), Planting (P), Year (Y), and interactions between these factors (Table 3.4 and 3.5). Despite the significance of the interactions, JR consistently had higher shoot yield than SQ at both planting dates and years of the study (Table 3.4) and JR root yield was higher than SQ root yield in all planting dates, except May 2008 (Table 3.5).These results are consistent with yield data from the colored plastic mulch experiment where JR yielded more root and shoot biomass than SQ (Chapter 2). Netting did not affect shoot yield (Table 3.4) and had an inconsistent effect on root yield across cultivars (Figure 3.1) and between planting dates and years of the study (Table 3.6). Although root yields were generally higher in no netting plots than those with netting, the difference was only significant in the August 2009 planting, but not in any other planting (Table 3.6). This is in contrast to previous research which reported that nettings increased fruit weight when compared to no netting controls (Elad et al., 2007; Retamales et al., 2008).

Glucosinolate profiles and concentrations. Total GSL concentrations were influenced by cultivar and tissue type with shoots having lower GSL concentrations than roots (Tables 3.4 and 3.5). Across both cultivars and tissue types, six GSLs: gluconapin

(GNP; 3-butenyl GSL), glucobrassicanapin (GBN; 4-pentenyl GSL), gluconapoleiferin (GF; 2-hyrdoxy-4-pentenyl GSL), progoitrin (PRO; 2-hydroxy-3-butenyl GSL), gluconasturtiin (GNS; 2-phenylethyl GSL), and 1-methoxyglucobrassicin (1MGB; 1-methoxy-3-ylmethyl GSL), comprised over 90% of the total GSL concentration. Therefore, the effect of netting, cultivar and planting environment on GSL concentration will only be discussed for these six GSLs.

In shoots, C was a significant factor for all individual GSLs but not total GSLs (TTL; Table 3.4). These results are consistent with data from Chapter 2 where TTL concentrations in JR and SQ shoots were not consistently different from each other. C x Y and C x P interactions were also significant for some individual GSLs. Despite these interactions, SQ shoots always had higher PRO, GF, GBN, 1MGB and GNS than JR and JR shoots had higher GNP than SQ. Netting was only a significant factor for GBN in shoots where no netting resulted in the highest GBN concentration (83.9 $\mu mol \cdot 100g^{-1}$ fw) and red netting resulted in the lowest GBN concentration (64.6 $\mu mol \cdot 100g^{-1}$ fw). N did not influence TTL in shoots. Planting date significantly affected TTL, with May plantings yielding approximately 50% more shoot TTL than August plantings (Table 3.4). This data is consistent with Charron and Sams (2004) who found that spring growth conditions resulted in higher GSL concentrations in *Brassica oleracea* than fall growth conditions. In their study, Charron and Sams (2004) concluded that the seasonal changes in GSL concentrations were not the result of one environmental variable such as temperature or photoperiod, but due to the interactive effects of several variables.

In root tissues, C, P, Y and interactions between these factors significantly influenced TTL, individual GSLs and GSL proportions (Table 3.5). The P X Y interaction resulted from the highest root GSL concentrations occurring in the May 2009 planting but the lowest root GSL concentrations occurring in the May 2008 planting in both cultivars (Figure 3.2). Despite the significance of these interactions PRO, GBN, GF and 1MGB, and TTL were always higher in SQ than JR roots and GNP was consistently higher in JR than SQ roots. These results are consistent with data from Chapter 2 where

SQ had higher TTL concentrations in root tissues than JR in most instances. Netting (N) had no significant effect on TTL or individual GSLs in roots (Table 3.5).

Year was a significant factor influencing GSL concentrations in roots and shoots. It is possible that climatic differences between the two years of the study contributed to the observed differences in GSL levels between 2008 and 2009 (Table 3.4 and 3.5). Indeed, TTL in shoots and roots was positively associated with climatic data (Table 3.3), particularly during 10 days before harvest. TTL in shoots was positively correlated with the mean maximum air temperature during the 10 days before harvest ($r=0.73$ and $r=0.74$, $p<0.001$, for SQ and JR, respectively). TTL in roots was also significantly correlated with harvest temperature ($r=0.62$, $p<0.001$ for JR and $r=0.36$, $p<0.05$ for SQ). This association is consistent with that reported by Charron et al. (2005) who noted a positive correlation between temperature 2-4weeks prior to harvest and GSL concentrations in several Brassica vegetables, but inconsistent with results from Chapter 2 where small negative correlations between GSL concentrations and climatic conditions prior to harvest were observed.

The fact that nettings had minimal or no effect on root and shoot GSL concentrations in turnip but that climatic conditions were strongly associated with GSL concentrations agrees with our previous work with colored plastic mulches where climatic factors and mulch-dependent soil temperatures were more strongly correlated with GSL concentrations than the changes in reflected light quality provided by the mulches. These findings suggest that currently available field treatments to alter light are not sufficient to consistently alter GSL concentrations. Although colored nettings and plastic mulches do alter the light quality surrounding the plant, it is possible that the effect of these treatments on GSL concentration is superceded by climatic conditions. Since climatic factors are so strongly correlated with GSL concentrations, future research should further examine the role of these climatic factors on GSL biosynthesis and regulation.

Table 3.1 Spectral properties of transmitted light measured 50cm below photoselective nettings.

Light Characteristic[1]	Photoselective Netting		
	Blue	Red	Yellow
PPF (400-700 nm)%	56	51	52
Blue (445-455 nm)%	63	26	0
Green (545-555 nm)%	68	61	74
Red (R; 640-650 nm)%	47	73	77
Far-red (Fr; 735-745 nm)%	0	0	12
R:Fr ratio[2]	1	1	0.9

[1]Values are expressed as percentages of incoming sunlight in the same wavebands which were assigned a value of 1.00. Calculations and descriptions adapted from those reported by Antonious et al.1996.
[2]R:Fr ratio acquired with a Skye R:Fr meter; R:Fr of incoming sunlight was 1.12.

Table 3.2 Mean soil temperatures 5cm below soil surface under photoselective netting treatments for May and August planting dates.

		Soil Temperature (°C)			
		Photoselective Netting			
Planting	Year	Blue	Red	Yellow	None
May	2008	18.9	19.5	19.0	19.5
	2009	19.3	19.4	19.6	19.6
August	2008	20.8	20.4	20.3	20.1
	2009	19.1	19.7	19.3	19.0

Table 3.3. Climatic data for each growing season and ten days prior to harvest for May and August planting dates.

Planting	Year	Mean Daily Maximum Air Temperature (°C)		Mean Daily Minimum Air Temperature (°C)		Cumulative Growing Degree Units		Mean Daily Solar Radiation (Langleys)	
		Season	Harvest	Season	Harvest	Season	Harvest	Season	Harvest
May	2008	23	27	11	14	694	190	519	558
	2009	23	29	12	17	710	224	506	544
August	2008	24	22	12	11	636	130	434	478
	2009	25	27	13	13	721	176	451	403

Table 3.4 Fresh weight yield, glucosinolate concentrations and proportions of turnip shoots across cultivars, photoselective netting treatments, planting dates and years.

Source of Variation[1]	Fresh Wt ($Mg \cdot ha^{-1}$)	Glucosinolate Concentration ($\mu mol \cdot 100g^{-1}$ fw)						
		PRO	GF	GNP	GBN	1MGB	GNS	TTL
Cultivar (C)								
Just Right	35±1	1±0	0±0b	121±7	61±4b	3±1	3±0b	191±12
Scarlet Queen	24±1	3±1	1±0a	63±4	88±5a	4±1	9±1a	175±12
Significance	*[z]	*[z]	*	*[z]	*	*[z]	*	NS
Netting (N)								
None	30±2	3±1	1±0	98±10	84±8a	3±1	7±1	200±18
Blue	28±2	2±0	0±0	95±11	74±7ab	3±1	6±1	184±19
Red	30±2	3±1	0±0	81±7	65±6b	4±1	5±1	162±13
Yellow	29±2	1±1	0±0	94±10	76±8ab	4±1	7±1	187±17
Significance	NS	NS	NS	NS	*	NS	NS	NS
Year (Y)								
2008	28±1	2±0	0±0	67±4	53±3b	1±0	2±0	127±6b
2009	30±1	2±1	0±0	117±7	97±5a	6±1	10±1	239±11a
Significance	*[z]	NS	NS	*[z]	*	*[z]	*[z]	*
Planting (P)								
May	26±1	3±0	0±0	108±16	90±13a	6±1	8±1	222±32a
August	32±1	2±0	0±0	76±11	59±9b	1±0	4±1	144±21b
Significance	*[z]	NS	NS	*[z]	*	*[z]	*[z]	*
Interactions:								
C X Y	NS	*	NS	*	NS	*	NS	NS
C X P	NS	NS	NS	*	NS	*	NS	NS
N X Y	NS	NS	NS	NS	NS	NS	NS	NS
N X P	NS	NS	NS	NS	NS	NS	NS	NS
P X Y	*	NS	NS	*	NS	NS	*	NS
P X C X Y	*	NS	NS	NS	NS	NS	NS	NS

[1]Means with different letters within each column and source of variation are significantly different at $P \leq 0.05$ as determined by Tukey's HSD. Progoitrin (PRO); Gluconapoleiferin (GF); Gluconapin (GNP); Glucobrassicanapin (GBN); 1-methoxyglucobrassicin (1MGB); Gluconasturtiin (GNS); Total (TTL).
Significance:[NS,*] Nonsignificant or significant at $P \leq 0.05$.

[z]Mean separations on main effects were not performed due to the presence of significant interactions.

Table 3.5 Fresh weight yield, glucosinolate concentrations and proportions of turnip roots across cultivars, photoselective netting treatments, planting dates and years.

Source of Variation[1]	Fresh Wt ($Mg \cdot ha^{-1}$)	Glucosinolate Concentration ($\mu mol \cdot 100g^{-1}$ fw)						
		PRO	GF	GNP	GBN	1MGB	GNS	TTL
Cultivar (C)								
Just Right	14±1	0±0b	0±0	84±3	33±1	70±3	6±1	200±9b
Scarlet Queen	12±0	4±1a	22±2	35±2	69±3	79±3	28±3	277±8
Significance	*[z]	*	*[z]	*[z]	*[z]	*[z]	*[z]	*
Netting (N)								
None	15±1	2±1	10±2	60±5	54±3	78±3	19±3	246±10
Blue	11±1	2±0	10±2	62±4	50±3	77±3	17±3	241±11
Red	13±1	2±0	13±3	58±4	50±3	73±3	17±3	236±9
Yellow	13±1	2±0	11±2	57±5	51±3	71±3	15±2	230±10
Significance	*[z]	NS	NS	NS	NS	NS	NS	NS
Planting (P)								
May	12±0	2±0	18±3	52±4	42±2	61±1	9±1	210±8
August	14±0	2±0	4±1	66±5	61±4	89±3	25±3	266±10
Significance	*[z]	NS	*[z]	*[z]	*[z]	*[z]	*[z]	*[z]
Year (Y)								
2008	12±0	3±1a	10±2	61±5	51±3	78±4	25±3	255±11
2009	14±1	1±0b	12±2	58±3	51±3	72±2	10±1	222±8
Significance	*[z]	*	NS	NS	NS	*[z]	*[z]	*[z]
Interactions:								
C X N	*	NS	NS	NS	NS	NS	NS	NS
N X P	*	NS	NS	NS	*	NS	NS	NS
C X P	NS	NS	*	*	*	*	*	NS
C X Y	NS	NS	NS	NS	NS	*	NS	NS
N X Y	NS	NS	NS	NS	NS	NS	NS	NS
P X Y	*	NS	NS	*	*	*	*	*
P X C X Y	*	NS	NS	*	NS	NS	NS	NS

[1]Means with different letters within each column and source of variation are significantly different at $P \leq 0.05$ as determined by Tukey's HSD. Progoitrin (PRO); Gluconapoleiferin (GF); Gluconapin (GNP); Glucobrassicanapin (GBN); 1-methoxyglucobrassicin (1MGB); Gluconasturtiin (GNS); Total (TTL).
Significance:[NS,*] Nonsignificant or significant at $P \leq 0.05$.

[z]Mean separations on main effects were not performed due to the presence of significant interactions.

Table 3.6 The effect of photoselective netting treatments on root yield between years and planting dates.

	Root Yield[1]			
	May		August	
Photoselective Netting	2008	2009	2008	2009
Blue	11±1a	9±1a	10±1a	13±1b
Red	10±0a	11±1a	11±2a	15±1ab
Yellow	14±1a	13±1a	14±1a	16±0ab
None	14±1a	15±2a	14±1a	17±1a

[1]Yield expressed as Mg·ha^{-1}f.w. Means with different letters within each column are significantly different at $P \leq 0.05$ as determined by Tukey's HSD. Lowercase letters represent significant mean differences between netting treatments within a planting date and year.

Figure 3.1. The interactive effects of cultivar and netting on root yield and total glucosinolate concentration in 'Just Right' and 'Scarlet Queen' turnips. Bars with different letters within each cultivar are significantly different at P<0.05 as determined by Tukey's HSD.

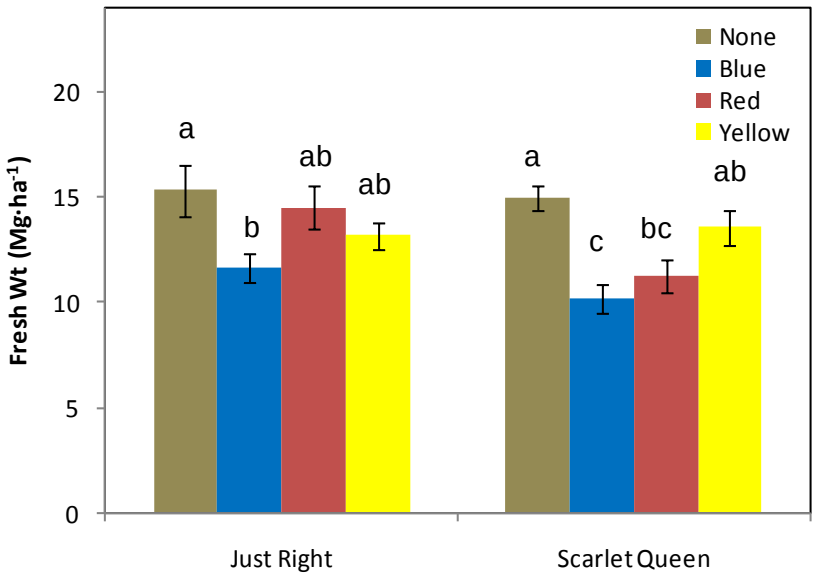
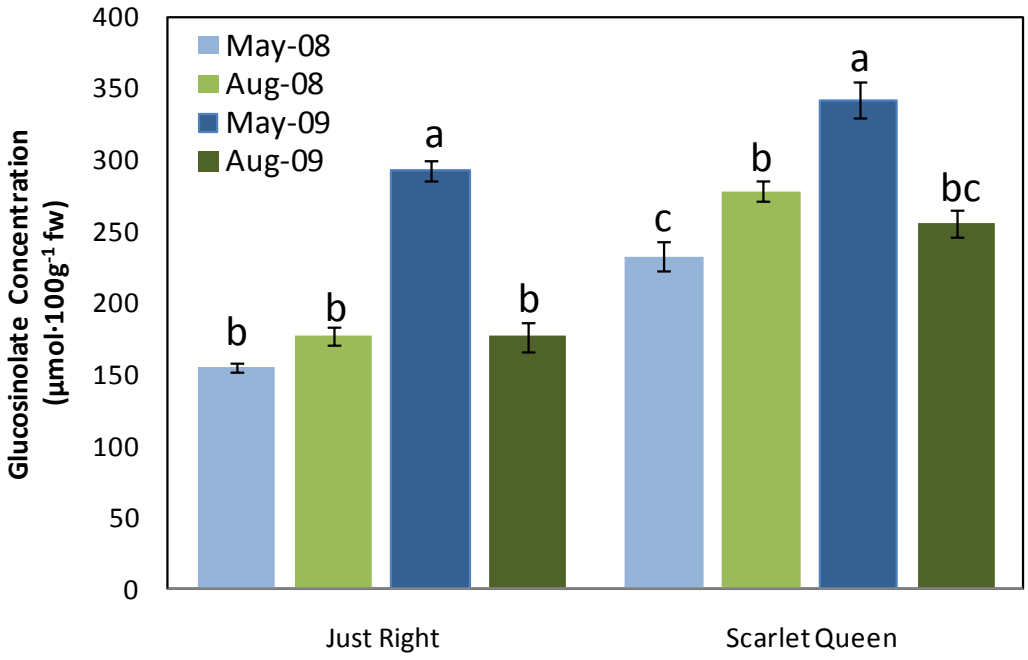


Figure 3.2 The interactive effects of year and planting date on total glucosinolate concentration in 'Just Right' and 'Scarlet Queen' turnip roots. Bars with different letters within each cultivar are significantly different at P<0.05 as determined by Tukey's HSD.



4 Temperature-induced glucosinolate accumulation is associated with expression of BrMYB transcription factors

4.1 Summary

Turnips produce glucosinolates (GSLs), thioglucosides whose hydrolyzed derivatives have been shown to provide chemopreventive benefits. Two cultivars of turnips ('Just Right'; JR and 'Scarlet Queen'; SQ) were grown under three different temperature regimes to assess the role of temperature on GSL production in roots and shoots. When compared to low temperature treatments, high temperature treatments increased total and individual GSLs in a tissue and genotype specific manner. When compared to low temperature treatments, total GSLs were approximately 70% and 130% higher on average in JR shoots and roots, respectively, grown at high temperature treatments. High temperatures also increased total GSLs in SQ shoots and roots by approximately 80% and 85%, respectively when compared to low temperatures. Gluconasturtiin (GNS, 2-phenylethyl GSL) concentration was inversely correlated with temperature with high temperature treatments resulting in 20% and 48% less GNS than low temperature treatments in JR and SQ roots, respectively. The indolic GSL, 1-methoxyglucobrassicin (1MGB; 1-methoxy-3-ylmethyl GSL) was the root GSL most elevated by increased temperature resulting in a 1000% increase on average in both cultivars between the low and high temperature treatments. These results show promise for the use of temperature to enhance the health promoting properties of turnip as 1MGB has potent chemopreventive effects. Gene expression analysis suggests that some BrMYB transcription factor expression levels are associated with temperature-dependent changes in GSL accumulation, however this association varies between cultivar and tissue type.

4.2 Introduction

Glucosinolates (GSLs) are thioglucosides found in Brassica vegetables that when hydrolyzed at the S-glucose bond create a suite of products involved in plant defense, flavor and human health. Consumption of vegetables containing glucosinolates has been

correlated with cancer and disease prevention (Hecht, 2000; Talalay and Fahey, 2001). Glucosinolates and their hydrolyzed derivatives, mainly isothiocyanates, have been shown to serve as chemopreventive agents by stimulating both protection from and excretion of carcinogens and by inhibiting tumor growth (Hecht, 2000; Neave et al., 2005). Identifying vegetable production systems that enhance GSL concentrations could have a significant impact on human health, particularly cancer prevention.

GSL concentration is highly influenced by genotype and environmental factors including soil fertility (Rosen et al., 2005), temperature (Charron and Sams, 2004) and light (Antonious et al., 1996; Charron and Sams, 2004; Engelen-Eigles et al., 2006). In our previous field experiments using colored plastic mulches and photoselective nettings at different planting dates we found strong correlations between temperature and solar radiation with GSL concentrations. We wanted to better understand the specific effect that the temperatures observed in our plastic mulch experiments would have on GSL concentration in a controlled environment. Our previous research using colored plastic mulches in a field setting demonstrated that plastic mulches both alter the light reflected into the canopy of turnip plants and also appreciably change soil temperature (see Chapter 2). Depending on the planting date and type of mulch, average soil temperatures under mulches during the turnip growing season can be up to 5°C above bare soil temperatures and 4°C below bare soil temperatures (Table 2.2). Temperature can have a significant impact on both shoot and root GSL concentration in a controlled environment. Charron and colleagues (2004) found that the concentration of the aliphatic glucosinolate gluconapin (GNP; 3-butenyl GSL) doubled in leaves and stems of *B. oleracea* plants grown at 32°C and the indolic glucosinolate 1-methoxyglucobrassicin (neoglucobrassicin; 1MGB; 1-methoxy-3-ylmethyl GSL) increased three-fold in roots grown at 32°C compared to those grown at 12°C (Charron and Sams, 2004). Engelen-Eigles and colleagues (2006) found that the concentration of the aromatic glucosinolate gluconasturtiin was increased in *Nasturtium officinale* grown at 10°C and 15°C as compared to that grown at 20°C or 25°C.

Both aliphatic and indolic GSL biosynthesis are regulated by various genetic components. In *Arabidopsis*, these regulatory elements can either repress GSL production, such as *SLIM1* (*Sulfur Limitation1*; Maruyama-Nakashita et al., 2007), promote GSL production such as *AtDof1.1* (DNA-binding-with-one finger; Skirycz et al., 2006) and subgroup 12 R2-R3 MYB transcription factors (Gigolashvili et al., 2009); or both positively and negatively regulate GSL production such as the nuclear-localized calmodulin-binding protein IQD1 (Levy et al., 2005). Two distinct clades of subgroup 12 R2-R3 MYB transcription factors regulate GSL biosynthesis and accumulation. Clade 1 members *MYB34*, *MYB51* and *MYB122* regulate indolic GSL biosynthesis while clade 2 members *MYB28*, *MYB29* and *MYB76* regulate aliphatic GSL biosynthesis (Gigolashvili et al., 2009). Members within each clade appear to have distinct, but overlapping roles. It has been suggested that *MYB34*, *MYB122* and *MYB 51* positively regulate glucobrassicin, but only *MYB51* positively regulates other GSLs such as methoxylated indolic glucosinolates (Gigolashvili et al., 2007). However, Malitsky and colleagues (2008) did not observe this trend and found that both *MYB34* and *MYB51* overexpression resulted in increases in 1-methoxyglucobrassicin.

MYB28 regulates both long (6-8 carbon length) and short-chain (3-5 carbon length) aliphatic GSL biosynthesis and its overexpression can result in increased accumulation of indolic GSLs (Hirai et al., 2007). *MYB29* and *MYB76* also regulate GSL biosynthesis to a lesser degree, but only short-chain aliphatic GSL biosynthesis (Sønderby et al., 2007; Gigolashvili et al., 2008). MYB transcription factors may also regulate GSL biosynthesis in a tissue-specific manner with *MYB34* and *MYB122* being preferentially expressed in root tissues and *MYB51* being preferentially expressed in leaf tissue (Gigolashvili et al., 2009). *MYB28*, *MYB29* and *MYB76* appear to be expressed to some degree in all tissues (Gigolashvili et al., 2009). In most cases, the expression pattern of MYB transcription factors correlates with expression of other GSL biosynthetic genes (Gigolashvili et al., 2009). No MYB transcription factors have been identified that specifically regulate aromatic GSL biosynthesis.

Using a comparative genomics approach, Zang and colleagues (2009) identified

GSL biosynthetic and regulatory orthologs in *Brassica rapa* (Br) with high sequence identity (72-94%) to *Arabidopsis thaliana* GSL biosynthetic genes. In most cases, multiple copies of these genes were found in the *B. rapa*. Two MYB transcription factor orthologs, *MYB76* and *MYB122*, were absent or non-functional in *B.rapa.* Br*MYB122-1* was found to be non-functional due to a deletion that resulted in a premature stop codon while *MYB76* was absent in *B.rapa* (Zang et al., 2009). The absence of *MYB76* in *B. rapa* is interesting because *MYB76* positively regulates methylsulfinylated short-chained aliphatic GLSs in *Arabidopsis* (Sønderby et al., 2010) and these GLSs are absent or in low concentrations in turnip (Carlson et al., 1987; Li et al., 2007; Smetanska et al., 2007). Although GSL associated MYB orthologs from *Arabipdopsis* have been identified in *B. rapa* through comparative genomics (Zang et al., 2009), there is little data on the influence of these MYBs on GSL accumulation in *B. rapa*. It is possible that tissue-dependent changes in BrMYB transcription may result in tissue-dependent changes in GSL accumulation observed in turnips grown at different temperatures.

Genotype can be a major factor in determining baseline GSL concentration in plant tissues. Rosen et al. (2005) found that red cabbage had inherently higher GSL content than green cabbage. Justen and Fritz found that roots of the red-skinned turnip cultivar 'Scarlet Queen' have inherently higher GSL levels than purple-crown and white-skinned turnip roots (Chapter 2 and 3). Genotypes can also vary in terms of GSL enhancement by environmental factors (Chapter 2 and 3; Rosen et al., 2005; Fritz et al., 2010). The objective of this experiment was to quantify shoot and root tissue GSL concentrations of two turnip cultivars 'Just Right' and 'Scarlet Queen' under three different temperature regimes representing the range of soil temperatures present in our previous plastic mulch experiment. We also sought to examine the relationship between temperature and GSL biosynthesis regulation by quantifying transcript levels of BrMYB orthologs in turnips grown at different temperatures. These MYB orthologs have been shown to regulate GSL biosynthesis in Arabidopsis. Our results show that a relationship does exist between temperature-induced GSL accumulation and some BrMYB transcript levels, but the relationship is not consistent across cultivars and tissue-types.

4.3 Materials and Methods

Growth chamber conditions and plant materials. Turnip seeds (cv.'Just Right'(JR; Jordan's Seed,Woodbury,MN) and 'Scarlet Queen'(SQ; Johnny's Seeds, Winslow, ME) were grown in a soilless media (Sunshine Grow Mix LC-8, Sun Gro Horticulture, Bellevue, WA) in 10cm pots. The plants were grown for 42 days under three different temperature treatments (Table 4.1) with a relative humidity of 75%. The photoperiod was 14hr with a constant PAR of ~475µmol·m^{-2}·s^{-1} and a R:Fr ratio of 2.1. Plants were grown in a model GCW-15 growth chamber (Environmental Growth Chambers, Chagrin Falls, OH) fitted with metal halide bulbs. Plantlets were thinned to one plant per pot one week after seedling emergence. Plants were watered daily and fertilized weekly with 200 ppm N using 20-10-20 Peat-lite Water Soluble Fertilizer (Scotts Miracle-Gro Company, Marysville, OH).

All sampling was carried out between 09:00-11:00 h. The experiment was replicated twice over time. JR and SQ plants were arranged in a completely randomized design of four replications within each growth chamber. Turnip plants were harvested six weeks after seeding and fresh weights were recorded after separation of shoots and roots. Turnip roots were 5-8 cm in diameter at the time of harvest. All samples were stored at 4°C prior to processing for GSL quantification and were processed within six hours after harvest.

Turnips used for gene expression analysis were seeded in a low and high temperature regime according to the previously described growth chamber experiment (Table 4.1). The light level was ~500 µmol·m^{-2}·s^{-1}. JR and SQ were seeded in three biological replications at ten day intervals. Samples from six week-old plants were harvested for gene expression analysis and immediately flash frozen and stored at -80°C until RNA extraction. A subsample of this plant material was also used for GSL quantification according to the method below.

GSL quantification. Extraction and GSL quantification was performed as per Hect et al., (2004), utilizing modifications from Rosen et al. (2005). Briefly, a 50g leaf sample or 100g root sample was boiled in 300mL of boiling water for 5 min to deactivate

myrosinase. Boiled samples were macerated in a blender for 2 min. A 40 mL-aliquot of blended sample was homogenized using a BioSpec M133 Homogenizer (BioSpec Products, Inc., Bartlesville, OK) set at 12,000 rpm for 2 min, then centrifuged for 10 min at 5000 *gn*, 4°C.

Desulfoglucosinolate (ds-GSL) extraction was performed using conditioned solid phase strong anion exchange (SAX) columns (Sigma-Aldrich, St. Louis, MO). Sinigrin (2-propenyl GSL; Sigma-Aldrich, St. Louis, MO) was added to the conditioned SAX columns as an internal standard. To desulfate, samples were incubated with two units (0.2mg/mL) of sulfatase (aryl-sulfate sulfohydrolase; EC 3.1.6.1; Sigma-Aldrich, St. Louis, MO) on SAX columns for ~15 hr at room temperature (~21°C) then eluted with 3mL water and the collected volume was determined by weight. Further washing of the columns yielded no additonal ds-GSLs confirming complete elution. Eluent was stored at -20°C until HPLC analysis.

HPLC analysis was performed on an Agilent 1200 Series Quaternary system (Agilent technologies, inc., Santa Clara, CA) set at λ=229 nm using a Luna C18, 5µm, 250 x 4.6 mm column (Phenomenex, Torrance, CA) set at 30ºC. A 50-µL aliquot of the eluent was separated on the system with a flow rate of 1.0mL·min^{-1} using the following gradient: solvent A= water and B= acetonitrile; 0 to 2 min, 95% A, 5%B; 2 to 20 min, 85% A, 15% B; 20 to 23min, 53% A, 47% B; 23 to 30 min, 0% A, 100% B; and 30 to 33 min, 95% A, 5% B. Peaks were integrated using ChemStation for LC 3D Systems, Rev. B.04.01 software. A sample chromatogram is presented in Figure S4. GSL peak identities were confirmed using retention time and Ultra Performance Liquid Chromatography-Mass Spectrometry (Waters Corporation, Milford, MA) using a C18 column, a water:acetontirile gradient and negative electrospray ionization (see Figure S5 for an example mass spectra). Ds-GSL concentrations were calculated using relative quantification with an internal standard (sinigrin) and previously published response factors (EU, 1990). Ds-GSL concentrations are reported on a µmol·100g^{-1} fw basis.

Quantitative PCR analysis. Frozen turnip leaf and root tissues were ground in liquid nitrogen then immediately used for RNA extraction with RNeasy Mini Kits and

on-column DNAse digestion to remove genomic DNA contamination (Qiagen, Inc., Valencia, CA). Total RNA was quantified using a Qubit™ fluorometer (Invitrogen Corp., Carlsbad, CA). Total RNA was reverse-transcribed into cDNA using the Super-Script III reverse transcriptase kit (Invitrogen). Quantitative PCR and transcript analyses were performed using a Bio-Rad CFX96™ Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA).

PCR reactions contained 10µl iQ™ SYBR Green Supermix (Bio-Rad), 1 µl each of 10 mM forward and reverse primer, 1 µl cDNA template and nuclease-free water to a final volume of 20 µl. A negative control (nuclease-free water) was included in each run. PCR primers were designed to amplify paralogs of a given gene family for *BrMYB28*, *BrMYB29*, *BrMYB34* and *BrMYB51* genes, and *BrActin1* using a combination of Primerblast and MEGA sequence alignment tool (version 4.1, Biodesign Institute, A240, Arizona State University, Tempe, AZ). Primers amplified a region containing at least one exon/intron split. Primer sequences are listed in Table S1. Sequence alignments between the sequenced PCR products for a given primer set and the intended gene to amplify are presented in Figure S9.

The PCR reaction was initiated with a denaturation step for 5min at 94°C, followed by 40 cycles of denaturation (94°C for 30s), annealing (depending on primer set, 50-54°C for 30 s) and extension (72°C for 30s). Fluorescence was measured at the end of each extension step. The PCR reaction was terminated with a final extension step for 7 min at 72°C then subjected to a melting curve analysis. The data were analyzed with CFX Manager software (Bio-Rad). Transcript levels were normalized to *Brassica rapa* Actin 1 (*BrACT1*, Genbank accession number FJ969844). *BrACT1* threshold cycle (Ct) values ranged between 19.1-22.6 across temperature treatments, tissues and cultivars. A standard curve was generated for each primer set using a cDNA serial dilution to calculate PCR efficiencies used for relative transcript level analysis. Error bars shown represent the means ± SE of three biological samples and three technical replicates (Figure 4.1).

Statistical Analysis. Data were analyzed with R 2.9.1 (R Foundation for Statistical Computing, Vienna, Austria). The significance of differences between treatments, cultivar and experiments was assessed by a fixed-factor ANOVA. Data are expressed as means. Mean values were considered significantly different at $P < 0.05$ as determined by Tukey's HSD. Student's t-test was used to determine significant differences in transcript accumulation between samples.

Light Measurements. PAR was measured with an Apogee Quantum Meter, model QMSW-SS (Apogee Instruments, Inc., Logan, UT), and R:FR ratios, were measured with a Skye red -far red meter (Skye Instruments, Ltd., Powys, UK).

4.4 Results and Discussion

Fresh weight. Cultivar by temperature interactions and temperature by experiment interactions were significant for fresh weight and several individual GSL concentrations, therefore data will be presented separately for experiments, cultivars and tissue types (Table 4.2 and 4.3). Temperature was a significant factor influencing root and shoot fresh weight in both JR and SQ (Table 4.2). In shoots, there was a significant reduction in shoot fresh weight in low and medium temperature treatments when compared to the high temperature treatments in experiment two but not experiment one (Table 4.2). In both experiments and both cultivars, the medium temperature treatment resulted in the highest root fresh weights (Table 4.2). JR and SQ roots accumulated about 45% less biomass in high temperature treatments than low temperature treatments (Table 4.2). The influence of temperature on root yield is important because biomass accumulation has been inversely correlated with GSL concentration (Antonious et al., 1996; Radovich et al., 2005; Rosen et al., 2005). Thus increasing temperatures may result in higher GSL concentration, yet lead to less GSL yield per plant. Despite the differences in fresh weight, GSL concentrations per root were still approximately 30% and 15% higher in JR and SQ roots, respectively, grown at the high temperature than at the low temperature.

Glucosinolate Concentration. Temperature had a significant effect on total GSL concentration (TTL) in shoot and root tissue of both JR and SQ (Table 4.3). In all cases,

shoot and root TTL was always significantly lower in the low and medium temperature treatments than the high temperature (Table 4.3). This temperature-induced increase in GSLs resulted in an approximately 180% increase in TTL concentration in roots and shoots grown in the high temperature treatment when compared to the low temperature treatment. Although high temperatures significantly reduced root yield, the high temperature treatment still resulted in increased TTL GSL per plant when compared to the low temperature treatment (356 versus 256 µmol/root for JR and 428 versus 369 µmol/root for SQ) suggesting that the increase in GSL concentrations resulting from high temperatures was not strictly due to a dilution effect.

Cultivars and tissues varied in individual glucosinolate concentrations and profiles. In shoot tissue, two GSLs, gluconapin (GNP; 3-butenyl GSL) and glucobrassicanapin (GBN; 4-pentenyl GSL) comprised ~95% of the total glucosinolate concentration. In root tissue, five GSLs, progoitrin (PRO; 2-hydroxy-3-butenyl GSL), GNP, GBN, 1-MGB and GNS, comprised ~90% of the total glucosinolate concentration. Therefore, the effect of temperature on individual GSLs will be discussed in detail only for these five GSLs. In JR root tissue, GNP was the dominant GSL, comprising ~50% of TTL across temperature treatments (Table 4.3). In SQ roots, the dominant GSL was dependent on the temperature treatment, with GNS being the predominant GSL (36% of TTL) in low temperatures, and PRO being the dominant GSL (32% of TTL) in high temperature. GSL profiles for JR and SQ are similar to those observed in our colored plastic mulch and netting experiments, however absolute GSL concentrations were much lower in the field experiments than the growth chamber experiments (~35% and ~60% less for shoots and roots, respectively; Chapter 2 and 3). Our results are also consistent with previous data establishing GNS to be the primary GSL and PRO to be the second most abundant GSL in turnip root tissue (Zhang et al., 2008) and GNP to be the primary GSL in turnip shoot tissue (Smetanska et al., 2007; Carlson et al., 1987).

The effect of temperature on individual GSLs varied depending on the cultivar, tissue type and experiment (Table 4.3). In JR shoots, GNP was the GSL most influenced by temperature treatment with the high temperature treatment increasing GNP by 182%

(266 μmol·100g^{-1} fw) and 140% (165 μmol·100g^{-1} fw) when compared to low and medium treatments, respectively. A similar trend was observed in JR roots and SQ shoots, but not SQ roots. This increase in GNP is similar to that found by Charron and colleagues (2004) who saw GNP double in leaves and stems of *B. oleracea* plants grown at elevated temperatures. In JR shoots, GBN also increased a similar percentage as GNP with temperature, however since GBN is lower in JR shoots, the absolute increases were not as large (54 and 29 μmol·100g^{-1} fw increase when compared to low and medium temperature treatments, respectively). PRO was consistently increased in SQ shoots in high temperature treatments, but not JR shoots. GNS concentration decreased with increased temperature in JR and SQ shoots. High temperature grown JR shoots had 75% less GNS than those grown at low temperature, however GNS is present at inherently low concentrations in turnip shoot tissues, thus these decreases had minimal effects on the profile. Shoot 1MGB concentration was not significantly altered by temperature in most instances.

Root tissue GSLs were also significantly impacted by temperature (Table 4.3). Although the high treatment resulted in dramatic changes in GSL content, the medium treatment led to minimal changes in most GSLs when compared to the low temperature treatment. In the high treatment, 1-MGB increased by 30 μmol in JR roots and 82 μmol·100g^{-1} fw in SQ roots, respectively. This amounts to an 1100% and 850% increase in 1-MGB for JR and SQ, respectively. This temperature-dependent increase in 1-MGB in root tissue was demonstrated by Charron and Sams (2004) who reported a three-fold increase in 1-MGB in roots grown at 32 °C compared to those grown at 12 °C. The fact that 1-MGB can be manipulated so drastically with environmental conditions speaks greatly to the role of cultural practices in yielding vegetables with great nutraceutical potential. The bioactive hydrolysis product of 1-MGB (NI3C) has been shown to have chemopreventive properties exceeding that of I3C, the bioactive hydrolysis product of glucobrassicin (Neave et al., 2005). Temperature-dependent increases in GNP, GBN and PRO were also observed in root tissues, but these varied with cultivar. In JR roots, GNP and GBN values were increased by 265% (145 μmol·100g^{-1} fw) and 195% (18

μmol·100g^{-1} fw), respectively. In SQ roots, temperature did not have a significant effect on GNP and GBN concentrations. PRO was increased between low and high temperature treatments in JR and SQ roots by 600% (43 μmol·100g^{-1} fw) and 398% (128 μmol·100g^{-1} fw), respectively.

In root tissues, GNS concentration decreased in the high temperature treatment. The extent of temperature-dependent GNS changes varied with cultivar and experiment. In JR roots, GNS concentrations were significantly decreased in the low temperature treatment in experiment one but not experiment two. In SQ roots, GNS concentrations were consistently reduced in high temperature treatments. This decrease in GNS is consistent with previous data showing an inverse relationship between temperature and GNS content (Engelen-Eigles et al., 2006). The variability in temperature-induced GNS concentrations between cultivars is consistent with previous work on cabbage varieties where GSL enhancement from varying fertility levels and jasmonic acid applications has been cultivar-dependent (Rosen et al., 2005; Fritz et al., 2010). It is possible that the glucosinolate profiles of some genotypes are more influenced by environmental treatments than others.

GSL regulatory gene transcript levels. One possible reason for the increases we observed in GSL accumulation at increased temperature may be due to changes in GSL biosynthesis. Previous research in *Arabidopsis* has shown that MYB transcription factors play a significant role in regulating GSL biosynthesis (Hirai et al., 2007; Gigolashvili et al., 2009). Using primers based on *B. rapa* GSL MYB orthologs, we performed quantitative PCR to examine the relationship between GSL regulatory gene transcript levels and temperature, and to determine if these transcript levels are associated with the GSL phenotype we observed at different temperature treatments.

We examined the regulatory genes *BrMYB28*, *BrMYB29*, *BrMYB34*, and *BrMYB51* in root and shoot tissues of two turnip cultivars grown at a high and low temperature treatment. Our results indicate that GSL MYB transcript levels are influenced by temperature and are associated with changes in GSL accumulation in some cultivars and tissues (Figure 4.1). In *Arabidopsis*, *MYB28* and *MBY29*, members of a

distinct clade of subgroup 12 R2R3 MYB transcription factors, have been shown to positively regulate aliphatic GSL production with *MYB28* having the stronger regulatory effect of the two MYBs (Hirai et al. 2007). Normalized *BrMYB28* transcript levels were higher in high temperature than low temperature treated plants (ratio >1) in all tissues but SQ roots. *BrMYB28* transcript levels agree with GSL concentration data as there was a significant increase in the aliphatic GSLs, GNP and GBN in all tissues grown in the high temperature treatment except SQ roots (Table 4.3). In SQ roots, GNP or GBN were not elevated with temperature treatment (Table 4.3) and *BrMYB28* transcript levels in SQ root tissue did not increase with temperature (Figure 4.1). Normalized Br*MYB29* transcripts levels followed a similar pattern as Br*MYB28* across the tissues and cultivars with the ratio being highest in JR roots and lowest in SQ roots. Unlike *BrMYB28*, *BrMYB29* levels appeared to be repressed by high temperatures as indicated by the ratio <1 for all samples except JR roots, thus there does not appear to be a consistent association between aliphatic GSL accumulation and *BrMYB29* transcript levels. A similar discrepancy between *MYB29* expression and GSL content was also observed in *Arabidopsis* where *MYB29* gene knock-down did not result in apparent changes in GSL content (Hirai et al., 2007). It is interesting that the aliphatic GSL, PRO, was significantly elevated at high temperatures in SQ root tissues, but neither *BrMYB28* nor *BrMYB29* transcript levels were increased in these tissues in the high temperature treatment. These results suggest that PRO may be under different regulatory control than GNP and GBN.

Indolic GSL accumulation has been shown to be under the control of coordinated activities of *MYB34* and *MYB51* (as reviewed by Gigolashvili et al., 2009). These two transcription factors belong to a separate clade of subfamily 12 R2R3-MYB transcription factors than *MYB28* and *MYB29*. In our study, Br*MYB34* transcript levels followed a similar pattern as Br*MYB28* and Br*MYB29* with JR roots having the highest ratio and SQ roots having the lowest ratio although the fold changes between these transcripts were quite different (Figure 4.1). The similarity of transcript level patterns between *BrMYB28* and *BrMYB34* supports previous research suggesting a positive correlation between expression levels of *MYB34* and members of the *MYB28* clade but a negative relationship

between *MYB34* and *MYB51* expression in *Arabidopsis* (Malitsky et al., 2008). Similar to *BrMYB28, BrMYB34* transcript levels were significantly elevated with temperature treatments in all tissues except SQ roots. These results suggest a positive association between GSL concentration in JR roots and shoots and SQ shoots and Br*MYB34* transcript levels. *MYB51* has been suggested to positively regulate methoxlyated indolic GSL biosynthesis in *Arabidopsis* (Gigolashvili et al., 2007). Although we did observe a significant increase in 1MGB with the high temperature treatment in roots but not shoots (Table 4.3), this GSL data was not associated with *BrMYB51* transcript levels as there was no significant change in *BrMYB51* transcript levels in roots between the two temperature treatments (Figure 4.1).

Previous investigations have suggested that cross talk occurs between the two clades of GSL MYBs so that relative changes in expression of *ATR1*- like clade members (*MYB34* and *MYB51*) are inversely correlated with relative changes in expression of *MYB28*-like clade members (*MYB28* and *MYB29*) in *Arabidopsis* (Malitsky et al., 2008; Gigolashvili et al., 2008). Our results partially support this claim as increases in relative transcript levels of *BrMYB34*, a member of the *ATR1*- like clade, corresponded in most cases with decreases in relative transcript levels of *BrMYB29*, a member of the *MYB28*-like clade.

4.5 Conclusions

Temperature significantly impacted GSL accumulation with increases in temperature resulting in increased TTL, aliphatic and indolic GSL concentrations and decreased GNS concentrations. GSL regulatory gene expression was differentially influenced by temperature treatment depending on the tissue type and cultivar with some positive associations existing between temperature and GSL regulatory transcript levels. These results further emphasize the importance of temperature on GSL accumulation. The fact that some *BrMYB* transcript levels were both positively associated with temperature and GSL concentrations in some turnip tissues suggest that temperature may play a role in regulating GSL biosynthesis in *B. rapa*.

Table 4.1 Temperature conditions used in growth chamber experiments. The photoperiod was from 06:00-20:00h.

	Temperature Treatment (°C)		
Time	Low	Medium	High
03:00 h	15	17	21
10:00 h	19	24	28
14:00 h	22	27	34
21:00 h	19	24	28

Table 4.2 Fresh weight and glucosinolate proportions of 'Just Right' and 'Scarlet Queen' turnip shoots and roots across experiments and temperature treatments.

			Fresh weight (g)[1,2]	
			Experiment	
Cultivar	Tissue	Temperature Treatment	1	2
Just Right	Shoot	Low	122a	110c
		Medium	122a	168b
		High	133a	224a
	Root	Low	216b	320b
		Medium	272a	370a
		High	108c	240c
Scarlet Queen	Shoot	Low	97a	87b
		Medium	83a	117b
		High	90a	183a
	Root	Low	206a	309a
		Medium	229a	348a
		High	94b	229b

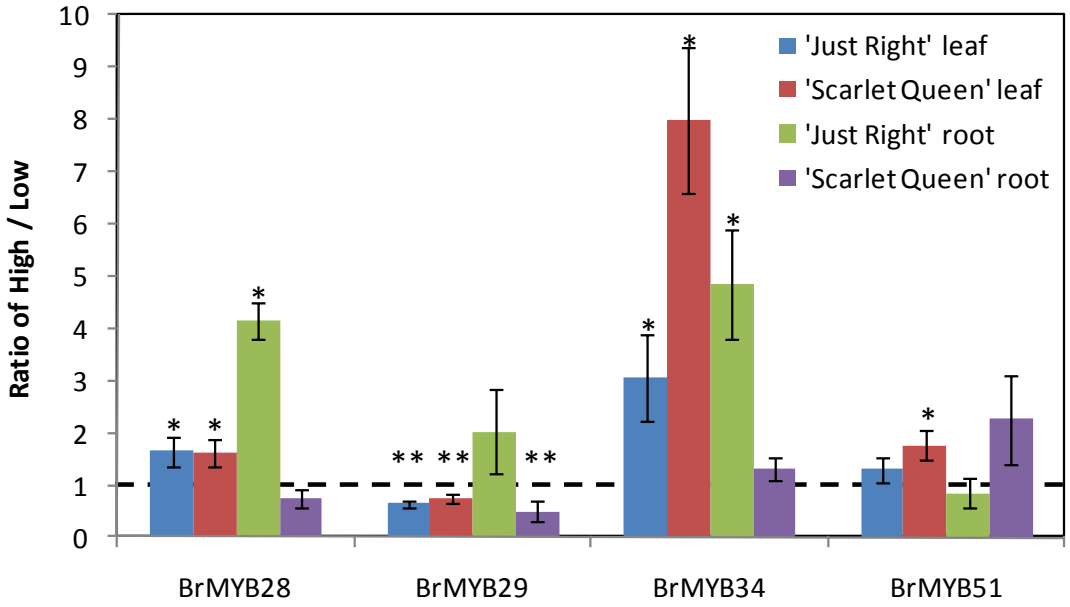
[1]Means with different letters within each column, cultivar and tissue type are significantly different at $P \leq 0.05$ as determined by Tukey's HSD.
[2] Fresh weight (g) determined from two turnip plants at time of harvest.

Table 4.3 Glucosinolate profiles of 'Just Right' and 'Scarlet Queen' turnip shoots and roots for both experiments and all temperature treatments.

Cultivar		Glucosinolate Concentration (μmol·100g^{-1} fw)											
		PRO		GNP		GBN		GNS		1MGB		TTL	
Tissue	Temperature Treatment	Experiment											
		1	2	1	2	1	2	1	2	1	2	1	2
Just Right													
Shoot	Low	0±0b	3±1a	279±13b	290±7b	94±4b	98±1b	4±0b	4±0a	0±0a	1±0a	394±19b	410±9b
	Medium	0±0b	1±0a	413±23b	358±26ab	116±6ab	127±11ab	1±0b	2±1a	1±0b	2±1ab	546±30b	503±35ab
	High	10±1a	2±1a	675±55a	425±22a	149±19a	151±13a	1±0a	1±0a	2±0b	2±0b	856±76a	609±35a
Root	Low	6±0b	10±1b	93±4b	83±6b	19±1b	20±1a	46±1b	55±3b	3±0a	2±0a	199±6b	183±10b
	Medium	15±1b	14±1b	104±5b	87±9b	19±1b	22±2a	31±2b	47±3b	6±0b	4±0a	209±4b	185±16b
	High	69±4a	33±3a	340±17a	126±5a	47±1a	27±1a	22±3a	59±2a	37±5b	28±3a	581±19a	294±4a
Scarlet Queen													
Shoot	Low	10±2b	9±1b	107±5b	122±8b	134±10b	191±12a	5±1a	16±1a	3±0a	6±2a	283±17b	358±21ab
	Medium	8±3b	5±1b	128±5b	97±10b	165±7ab	158±9a	2±0a	12±2a	2±0ab	5±1a	334±12b	290±19b
	High	48±4a	25±6a	238±9a	180±16a	209±16a	195±14a	1±1a	5±1a	3±0b	5±1b	534±27a	424±35a
Root	Low	43±4b	42±2b	38±8a	23±2a	73±11a	62±2a	106±10b	99±4b	14±3a	7±1a	311±46b	263±5b
	Medium	81±3b	58±4b	43±12a	19±2a	58±3a	58±2a	87±5b	92±2b	23±1a	19±2a	340±11b	284±9b
	High	210±41a	131±4a	129±59a	26±1a	82±13a	58±1a	41±6a	67±2a	112±24b	74±7b	648±38a	416±15a

[1]Means with different letters within each column, cultivar and tissue type are significantly different at $P \leq 0.05$ as determined by Tukey's HSD. Progoitrin (PRO); Gluconapin (GNP); Glucobrassicanapin (GBN); 1-methoxyglucobrassicin (1MGB); Gluconasturtiin (GNS); Total (TTL).

Figure 4.1 Transcript levels of turnip glucosinolate regulatory genes determined by real-time PCR. Ratio values are the fold difference between the normalized transcript levels (relative to actin) in 'Just Right' and 'Scarlet Queen' turnip tissues grown in high and low temperature regimes. Error bars are SE. **,* represent ratios significantly less than one or greater than one, respectively, at p<0.07.



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6 Appendix. Supplemental tables and figures

Figure S1. Reflectance properties of colored plastic mulches.

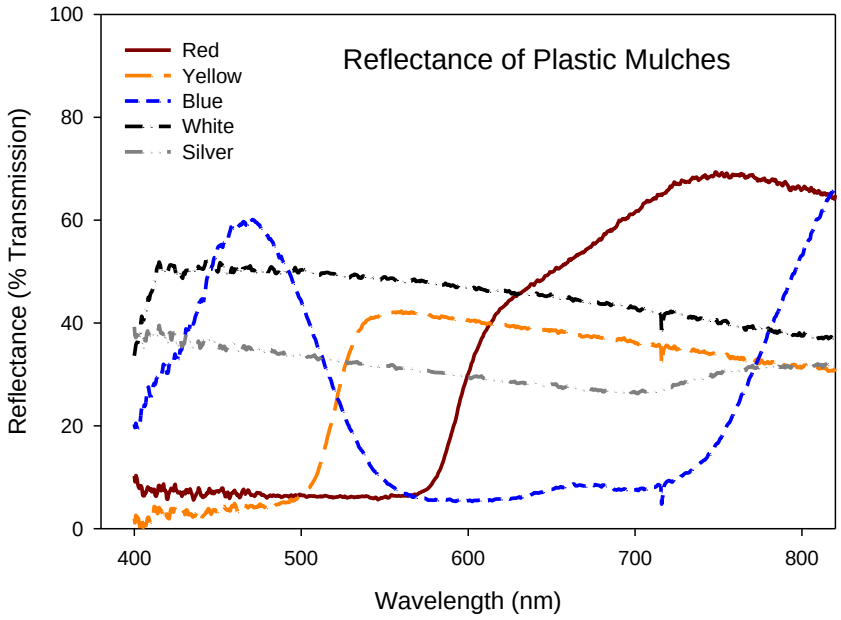

Figure S2. Turnip plants emerging from plastic mulch treatments.

Figure S3. Mean diurnal soil temperatures of plastic mulch treatments from May and August plantings. Soil temperatures were measured 5cm below the soil surface and averaged across 2006 and 2007 for each planting date.

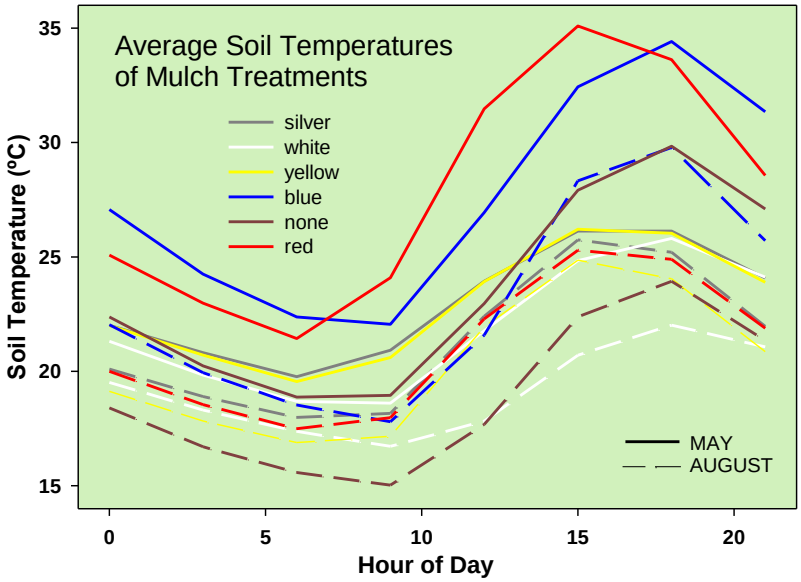

Figure S4. High performance liquid chromatogram of a representative turnip sample subject to desulfoglucosinolate extraction.

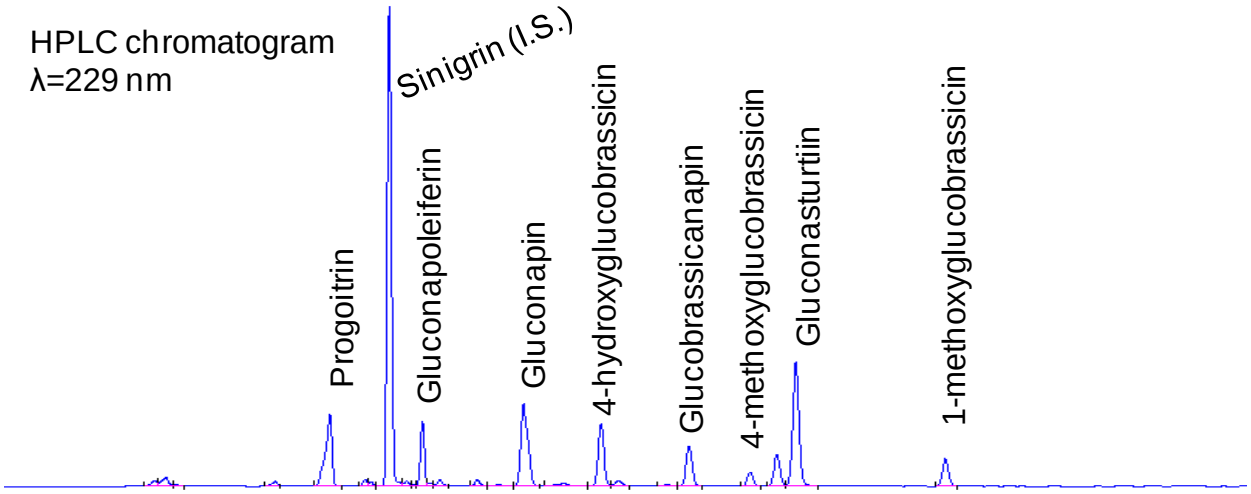

Figure S5. Mass spectra of a select desulfoglucosinolates from a representative desulfated turnip sample. Individual glucosinolates were separated by liquid chromatography prior to mass spectrometry. Mass spectra (Scan ES-) are shown for gluconasturtiin (M-H=342), glucobrassicin (M-H=367) and glucobrassicanapin (M-H=306).

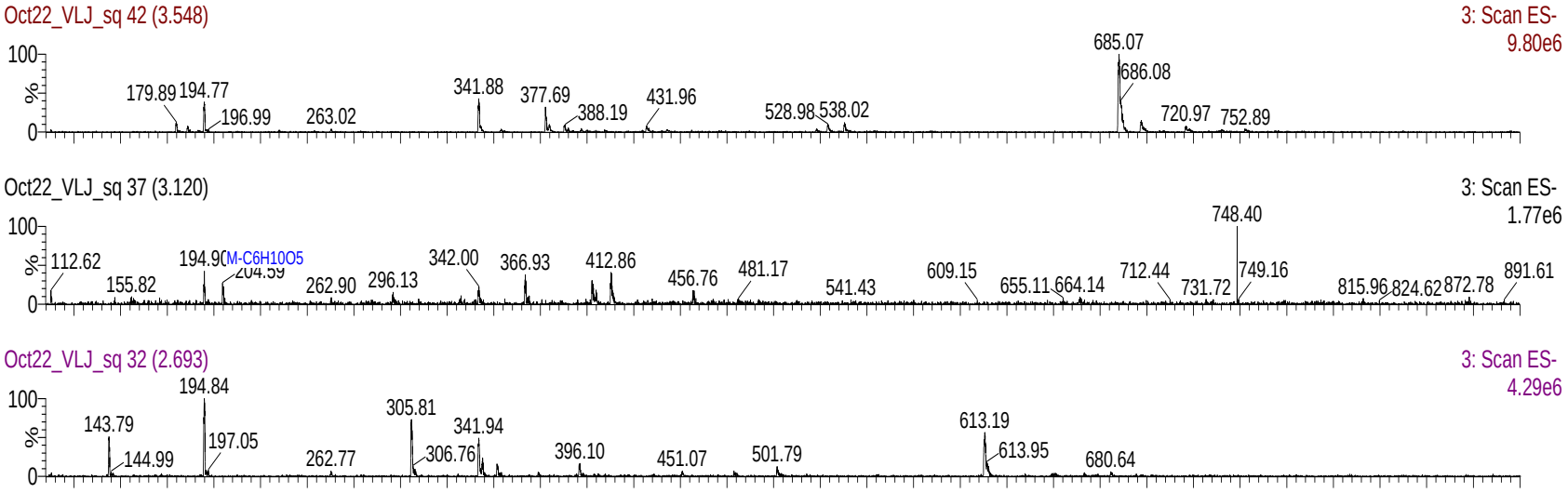

Figure S6. Spectrum of light transmitted through red, yellow and blue photoselective nettings.

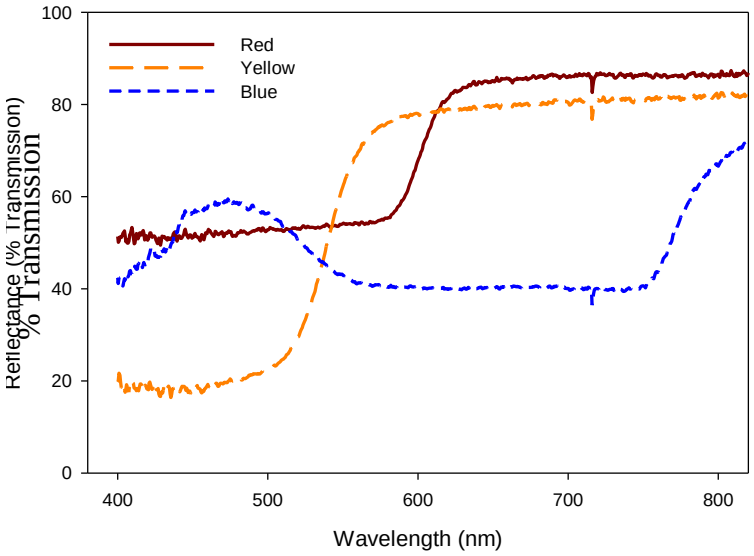

Figure S7. Turnip plants growing under photoselective netting treatments.

Figure S8. Mean diurnal soil temperatures of photoselective netting treatments from May and August plantings. Soil temperatures were measured 5cm below the soil surface and averaged across 2008 and 2009 for each planting date.

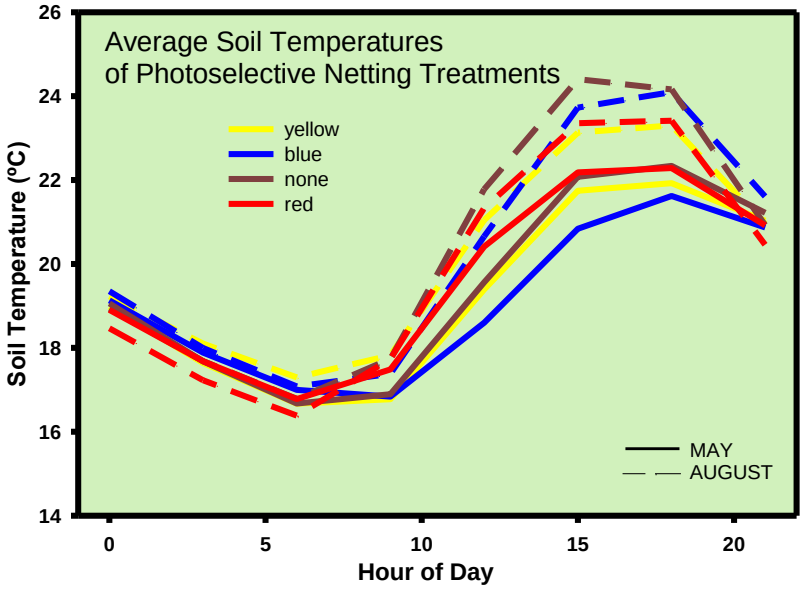

Figure S9. Clustal W alignments between sequenced PCR products from turnip cDNA amplified by primers listed in Table S1 and published *Brassica rapa* gene sequences.

Actin1

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PCR product         ATTCGTCTGCTCATGCCTGCTTTCC-TAGTGTTGTTGGTAGGCCAAGACATCATGGTGTC
Br_Actin1           -ATGATGCTCCCAGGGCTGTTTTCCCTAGTGTTGTTGGTAGGCCAAGACATCATGGTGTT
                      *  *   * ** * *** ***** ********************************
PCR product         ATGGTTGGGATGAACCAGAAGGATGCCTACGTTGGTGACGAAGCA
Br_Actin1           ATGGTTGGGATGAACCAGAAGGATGCCTACGTTGGTGACGAAGCA
                    *********************************************
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BrMYB28

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BrMYB28-2           GGGCATGGACCACCGAAGAAGATA-AGAAACTCATCTCTTACATCCACGAACATGGAGAA
BrMYB28-3           GTGCATGGACCACCGAGGAAGACA-AGACGCTCATCTCTTACATTCACGAACATGGAGAA
BrMYB28-1           GGGCATGGACCACCGAAGAAGACA-AGAAACTCATCTCTTACATCCATGAACATGGAGAA
PCR product         GGGCATGGA-CACCGAAGA-GACA--GAAACTCATCTCTTACATCCATGAACATGGAGAA
                    * ******* ****** ** ** *  **  ************** ** ************
BrMYB28-2           GGAGGCTGGCGCGACATTCCCCAAAAAGCTGG-TT
BrMYB28-3           GGTGGCTGGCGCGACATTCCCCAAAAAGCTGGGTT
BrMYB28-1           GGGGGATGGCGCGACATTCCTCAAAAAGCTGG-TT
PCR product         GGGGGATGGCGCGACATTCCTCAAAAAGCTGGGTT
                    ** ** ************** *********** **
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BrMYB29

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PCR product         ATGGACGGAATCAAGAGAGGAGAATTTAGCTATGAGGAAGAGCAAATCATTATCATGCTT
BrMYB29-1           AAACCCGATATCAAGAGAGGAGAATTTAGCTATGAGGAAGAGCAAATCATTATCATGCTT
BrMYB29-2           AAACCCCATATAAAGAGAGGAGAGTTTAGCTACGAGGAGGAGCAGATTATCATCATGCTT
                    *    *   ** *********** ******** ***** ***** ** ** *********
PCR product         CACGCTTCCCGTGGCAACAAGTGGTCGGTCATAGCAAGACATTTGCCCAAAAGGACAGAC
BrMYB29-1           CACGCTTCCCGTGGCAACAAGTGGTCGGTCATAGCAAGACATTTGCCCAAAAGGACAGAC
BrMYB29-2           CATGCTTCCCGTGGCAACAA-----------------------------------------
                    ** *****************
PCR product         AACG
BrMYB29-1           AACG
BrMYB29-2           ----
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BrMYB51

```
PCR product         AGGAGAGTTCACTGAAGATGAAGAACGTTCTATCATCTCTCTTCATGCCCTTCATGGCAA
BrMYB51-2           AGGAGAGTTCACTGAAGATGAAGAACGTTCTATCATCTCTCTTCATGCCCTTCATGGCAA
BrMYB51-3           ---AGAGTTCACTGAAGATGAAGAACTTTCTATCATCTCTCTTCACGCCCTTCACGGCAA
BrMYB51-1           AGGAGAGTTCACTGAAGACGAGGAACTTTCAATCATCTCTCTTCACGCTCTTCACGGCAA
                       *************** ** **** *** ************** ** ***** *****
PCR product         CAAATGGGCTGCAATAGCTCGTGGATTACC
BrMYB51-2           CAAATGGGCTGCAATAGCTCGTGGATTACC
BrMYB51-3           CAAATGGTCTGATATAGCTCGTGGATTACC
BrMYB51-1           CAAATGGGCTGCAATAGCTCGTGGATTACC
                    ******* ***  *****************
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Table S1. Forward and reverse primers used in real-time PCR analyses. Primers were designed to amplify all paralogs listed below.

Gene identifier (Genbank)	Name	Forward primer	Reverse primer
FJ584287	*BrMYB28-1*	AAGAAAGCCATGTTGTGTCG	TTTCCACACCTTTTCAACCC
FJ584288	*BrMYB28-2*		
FJ584289	*BrMYB28-3*		
FJ584290	*BrMYB29-1*	AGTTGTAGATTGCGATGGGC	CGTTGTCTGTCCTTTTGGGC
FJ584291	*BrMYB29-2*		
FJ584293	*BrMYB34-1*	ACTCTCCCGGAAAAAGCTGGAT	CGTTATCAGTTCGTCCAGCCA
FJ584294	*BrMYB34-2*		
FJ584295	*BrMYB34-3*		
FJ584296	*BrMYB51-1*	AGATGTGGCAAAAGCTGCAGA	GGTAATCCACGAGCTATTGCA
FJ584297	*BrMYB51-2*		
FJ584298	*BrMYB51-3*		
FJ969844	*BrActin 1*	ACCGGAATGGTCAAGGCTGGT	TGCTTCGTCACCAACGTAGGCA