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The effect of differences in aliphatic glucosinolate concentrations in *Arabidopsis thaliana* on herbivores of different feeding guilds and different levels of specialization

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Study programme: *Biology*
Period: *July-December 2010*
Thesis/Internship ENT
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Abstract

Members of the Brassicaceae family produce glucosinolates which act as defensive chemicals against herbivorous insects. Trichomes, on the other hand, can act as a physical defense against herbivores. Some specialist herbivores, like the phloem feeding aphid *Brevicoryne brassicae* and the leaf chewer *Pieris rapae*, have a way to by-pass the toxic effects induced by glucosinolates. Generalist insects, like the leaf chewer *Spodoptera exigua*, do not have a specific defense against glucosinolates.

In this research the effect of differences in glucosinolate concentrations on the performance of the three herbivores previously mentioned was tested. The performance was tested by rearing the insects on four *Arabidopsis thaliana* genotypes, each differing in glucosinolate concentration and/or trichome density.

It was found that differences in glucosinolate concentrations among the genotypes affected the performance of the herbivores. *Brevicoryne brassicae* was positively affected by higher glucosinolate concentrations. *Pieris rapae* experienced lower weight due to higher glucosinolate concentrations, probably mainly because of indole glucosinolates, and the caterpillar seemed to experience slower development due to trichomes. The generalist *S. exigua* also seemed to experience lower weight due to glucosinolates, more likely the aliphatic glucosinolates, and the caterpillar also seemed to have developed slower due to trichomes. Overall differences in aliphatic glucosinolates and trichome density do have an effect on herbivores. These effects seem to be specifically depending on the feeding guild and level of specialization.

Glucosinolates can be induced by herbivory, the concentrations in plants that are under herbivore attack should be analyzed and compared to the performance of the particular insect.

Preface

As an ecologist I was always fascinated by the vast amount of interactions among different species within an ecosystem. Most of all the large amount of solutions that species have to cope with different problems and other species that try to bypass these solutions amazes me.

In this research two different species have found two entirely different methods to cope with the same problem. With genetic engineering their problem will be enhanced and the effect this has for them will be examined.

I would like to thank Martine Kos and Joop van Loon from the Entomology chair group of Wageningen University for supervision and support during this project.

Contents

Introduction

Why is this research important?

As the food demand in the world keeps on growing, the importance of agricultural efficiency increases. Pesticides are used to protect crops from e.g. herbivorous insects. These pesticides often harm the environment and can be unsafe for human consumption, alternatives are of great importance (Romeis *et al.* 2006).

In an ecosystem there are a large number of interactions among species. The impact on one species can have a large impact on another species. These interactions are of large interest for researchers. When the first pesticides where introduced, they had a broad effect on most of the species and were also accumulative, therefore often having greater effect on top predators (Uno *et al.* 2001). Modern pesticides are much more specialized on fewer species, have a lower half-life and are used dependently on the number of herbivores present (Rohr *et al.* 2006). Still pesticides tend to lower the abundance of species and are likely to totally remove rare species (Desneux *et al.* 2006).

One of the pesticides used was *Bt* toxin, extracted from the bacteria *Bacillus thuringiensis.* This toxin targets specific species in specific taxonomic groups. It is applied by spraying it on the plants, where there is a chance it gets in contact with non-target species, or does not come in contact with target species. By genetically modifying the *Bt* gene into the crop, the crop could express *Bt* toxin on its own, increasing the chance the target species ingests the toxin and that non-target species that do not feed on the plant do not ingest the toxin (Dutton *et al.* 2003).

Bt transgenic crops target specific species within the Dipteran, Lepidopteran and Coleopteran insect orders (Groot & Dicke 2002). The reason for this is that the *Bt* toxins need specific receptors for binding. If a species does not have these receptors, it cannot be directly affected by the *Bt* toxin, resulting in a high specificity of the toxin to certain insect orders (Romeis *et al.* 2006). In US and China cotton, there was a decrease of 60-80% insecticide use against budworm-bollworm after they started using *Bt* transgenic crops (Romeis *et al.* 2006). Using genetically modified crops for pest control is more environmental friendly than relying on pesticides for pest control (Chen *et al.* 2008). Newly engineered crops, however, still need to be tested for their potential negative effects in an ecosystem.

In the plant family Brassicaceae, compounds known as glucosinolates confer resistance against herbivore attack. Glucosinolates are known to have a negative effect on herbivore generalists, but have limited to no negative effect on herbivore specialists (Hopkins *et al.* 2009). Some herbivore specialists are able to use the glucosinolates obtained when feeding from a plant, against their own natural enemy (Hopkins *et al.* 2009, Kazana *et al.* 2007). When the glucosinolate concentrations in a plant are increased by genetic modification, effects may be expected on the insect directly feeding on the plant. However, perhaps greater effects can be expected on higher trophic levels such as predators and parasitoids.

This paper describes research done with genetically modified brassicaceous species *Arabidopsis thaliana.* The gene MYB28, which is a key transcription factor in the synthesis of aliphatic glucosinolates, was inserted into the genome resulting in an increased production of aliphatic glucosinolates. The effect of the difference in aliphatic glucosinolate production on two specialist herbivores was tested. The performances of the cabbage aphid,

Brevicoryne brassicae, and the performance of the small white, *Pieris rapae*, were measured while feeding on three different *A. thaliana* wild-type genotypes and a modified MYB28 genotype of one of these genotypes. Furthermore, to test for effects of the genetic modification on generalist herbivores, the performance the beet armyworm, *Spodoptera exigua* was tested when fed with the MYB28 and wild-type *A. thaliana* genotypes.

Elaboration and information

Direct and indirect plant resistance

Plants can protect themselves against herbivory by direct and indirect resistance. Examples of direct resistance are toxins produced by the plant, wax coating on the leaf, trichomes and spines. Besides direct resistance, where the plant influences the herbivore directly, there is also indirect resistance. When a plant gets damaged it can emit volatiles into the air. These volatiles can act as attractant for predators and parasitoids of the herbivore causing the damage (Cortesero *et al.* 2000). They are therefore seen as 'a cry for help' from the plant (Dicke 2009, Degenhardt *et al*. 2003).

Plants of the *Brassicaceae* family have trichomes as their most important morphological resistance against herbivory (Traw & Dawson 2002). As chemical resistance these plants produce glucosinolates. These chemicals not only have toxic effects on the herbivores themselves, but also act as volatiles, attracting predators and parasitoids (Hopkins *et al.* 2009).

Glucosinolates

Glucosinolates (GLS) are organic compounds containing sulfur and nitrogen and are found mainly in the Brassicaceae plant family. They are important for humans as they play an important role in the strong flavors of e.g. broccoli, cabbage and mustard. Beside their flavor they also seem to have cancer prevention characteristics (Halkier & Gershenzon 2006).

The stable glucosinolate structure can be hydrolyzed by the enzyme myrosinase (Halkier & Gershenzon 2006, Hopkins *et al.* 2009). Myrosinase is stored in special cells, separately from glucosinolates, which are stored in vacuoles. When plant tissue damage occurs, e.g. by herbivore feeding, myrosinase comes into contact with glucosinolates (Fahey *et al.* 2001). When glucosinolates are hydrolyzed by myrosinase the products are a larger concentration of isothiocyanates and a smaller concentration of nitrile. These chemicals are toxic to a large variety of insects. The effects can vary between growth inhibition, feeding deterrence or downright toxicity (Halkier & Gershenzon 2006). Little is known about the mechanism in which these chemicals utter their toxicity, although isothiocyanates are known to react with sulfhydryl and amino groups of proteins in vitro (Halkier & Gershenzon 2006). For certain insect species isothiocyanates are more toxic than nitriles, e.g. for the larvae of *P. rapae* and *Trichoplusia ni* (Wittstock & Halkier 2002).

Epithiospecifier proteins (ESPs) are needed for the formation of nitriles instead of isothiocyanates (Mumm *et al.* 2008, Halkier & Gershenzon 2006). In a study were plants expressed ESPs, more nitriles were produced at the expense of isothiocyanates. These plants were less resistant against two herbivore generalists (Pope *et al.* 2008).

There are different kinds of glucosinolates. They can be distinguished by the rest (R) group, which consist of one of eight amino acids. Glucosinolate can be classified into three classes. The distinction depends on the amino acid precursor of the R group. Glucosinolates with an R group based on alanine, leucine, methionine, isoleucine and valine are grouped into aliphatic glucosinolates. Glucosinolates with an R group based on tyrosine and phenylalanine are grouped into aromatic glucosinolates, and glucosinolates with an R group based on tryptophan are grouped into indole glucosinolates (Fahey *et al.* 2001).

Jensen *et al.* (2010) tested the toxicity of both benzyl glucosinolates and the isothiocyanates the hydrolysis of these benzyl glucosinolates give. Benzyl glucosinolates were found to be toxic, but 10 fold less toxic than the isothiocyanates they produce after hydrolysis.

When glucosinolates are hydrolyzed by myrosinase, the end products can have direct toxic effects on insects and animals. Indole glucosinolates are more instable than aliphatic and aromatic glucosinolates. Indole glucosinolates decompose spontaneously, without myrosinase, into toxic hydrolysis products. Indole-3-carbinol can form a dioxin-like chemical when it comes into contact with acids in the stomach. This dioxin-like chemical is toxic for herbivore insects (Fahey *et al.* 2001, Kim & Jander 2007).

There are not many differences in function known between aliphatic and aromatic glucosinolates. Black vine weevil, *Otiorhynchus sulcatus*, is a generalist chewer of over 100 plant species. Toxicity of six different isothiocyanates to the eggs of *O. sulcatus* was tested. Aromatic isothiocyanates were more toxic to the eggs than aliphatic isothiocyanates, however, the reason for this is unclear (Borek *et al.* 1998).

Glucosinolates as volatiles

When plants get damaged by herbivore attack, volatile organic compounds can be detected in the air. These volatiles are thought to work as an indirect resistance mechanism against herbivore and pathogen attacks. Predators, parasitoids and herbivores can detect these volatiles and react to them in the form of attraction or repellence (Dicke 2009, Degenhardt *et al.* 2003).

Besides conferring direct resistance against herbivores, glucosinolates can also act as volatile cues for natural enemies of the herbivores attacking the plant, thereby conferring indirect resistance against herbivores. Nitriles and isothiocyanates that are formed from aliphatic and aromatic glucosinolates are known to act as volatile cues for natural enemies. However, the breakdown products from indole glucosinolates have no volatile characteristics (Hopkins *et al.* 2009, Pope *et al.* 2008).

An example of volatiles being used by parasitoids can be found in *Diadegma semiclausum.* When given the choice between plants infested with *Plutella xylostella* larvae and uninfected plants, the infested plants were preferred significantly. Their choice was probably based on volatile detection (Bukovinszky *et al.* 2005). Bukovinszky *et al.* (2005), however, did not examine the volatiles used by *D. semiclausum.*

Besides providing cues for natural enemies of herbivores, the herbivores themselves can also use the volatiles as cues for finding a host plant. For oviposition, the moth *P. xylostella* uses volatiles produced by the plant for locating a good site to deposit eggs on. Among these volatiles are glucosinolate hydrolysis products. A plant infested with *P. rapae* was more attractive for *P. xylostella* to oviposit on than the undamaged control plant. The assumed reason for this attraction was that *P. xylostella* larvae are more protected when *P.*

rapae larvae are also present, as the parasitoid wasp may have more trouble finding its host *P. xylostella* if the non-host *P. rapae* is also present (Shiojiri *et al.* 2001).

Induction of glucosinolates by insect herbivory

Glucosinolates are not only constitutively (i.e. independent of an attacker) present in plants, but are also induced when plant tissue is damaged. In an experiment by Mewis *et al.* (2005), the total glucosinolates content of *A. thaliana,* accession Col-0 was measured, after it was infested with the caterpillar *S. exigua* and the aphids *B. brassicae* and *Myzus persicae.* After the aphids fed for one week on the plants, the total glucosinolates levels increased by 16%-18% compared to control plants. The caterpillars on the other hand increased the total glucosinolates content by 2 fold in one day. Mainly short chained aliphatic glucosinolates increased with both species groups (Mewis *et al.* 2005). In 2006 Mewis *et al.* found that there was also an increase of indole glucosinolates levels after *M. persicae* had been feeding on Col-0 plants.

In general, indole glucosinolates are induced most often. The levels of induction range from 1.2- to 20-fold (Textor & Gershenzon 2009). Aliphatic and aromatic glucosinolates can increase by 1.2- to 3-fold, but are also reported to decrease after herbivore damage. This decrease tends to happen more often when the plant is attacked by a specialist herbivore. There was a 60% decrease of aromatic glucosinolates when the specialists *Delia radicum* and *Delia floralis* fed on cabbage. Meanwhile, the indole glucosinolates increased significantly. The suggested reason for this difference in increase of the different glucosinolate groups was that specialist herbivores have found ways to bypass the toxic effects of aliphatic and aromatic glucosinolates, but not the indole glucosinolates (Textor & Gershenzon 2009).

Plants seem to recognize the herbivore species feeding on them. When *P. rapae* fed on *A. thaliana* aliphatic glucosinolates did not increase, indole glucosinolates however did increase by 20%. When *S. exigua* was feeding three days on the same plant species, indole glucosinolates did not increase, but aliphatic glucosinolate concentrations in the plant increased by 50% (Mewis *et al.* 2006).

Glucosinolates are also induced when the aphid *M. persicae* is feeding on *A. thaliana.* De Vos & Jander (2009) treaded *A. thaliana* plants with saliva extracted from these aphids, the same induced glucosinolates were observed. After genetic analysis they found the 26 genes were activated after adding the saliva, some responsible for production of certain glucosinolates. The activation of these genes, which only activate after the plant comes into contact with aphid saliva suggest that *A. thaliana* induces different glucosinolates depended on the herbivore (De Vos & Jander 2009).

Trichomes

On the leaves of *A. thaliana*, trichomes can be found which act as a line of defense against herbivores (Larkin et al. 1996). These trichomes are formed on the epidermis of the plant and consist of single cell which is non-glandular. Development starts at the tip of the leaf and expands basipetally. The growth of more trichomes is limited when the leaf starts to stretch (Hülskamp & Schnittger 1998). In *A. thaliana* the trichomes are normally branched, having 2 or 3 branches (Gao et al. 2008).

It was found that in wild *A. thaliana* populations, the plants with higher trichomes density received less herbivore damage (Mauricio 1998). In nature, herbivores cause

favoring selection for increased glucosinolate concentrations and higher trichomes density. When herbivores are absent this pressure is lost and more plants with decreased trichomes density are found (Mauricio & Rausher 1997)

Agren & Schemske (1993) tested the difference in plant area damage of *P. rapae* on *Brassica rapa* with low versus high trichomes density. It was found that *P. rapae* causes significantly more damage on *B. rapa* when trichomes density on the plants is lower.

Trichomes can also be induced by herbivores damaging the plant. When cabbage was damaged by *P. rapae* (a specialist chewer) or by *T. ni* (a generalist chewer) the density of trichomes increases with 76% on the seventh leaf for *P. rapae* whereas the seventh leaf of the control and *T. ni* damaged plant did not differ. Plants damaged by *T. ni* had a 113% increase of trichomes on the ninth leave, whereas the ninth leave from the control and the plants damaged by *P. rapae* did not differ. No trichome increase is observed when cabbage is damaged by *Phyllotreta cruciferae*, a specialist pith maker (Traw & Dawson 2002).

Research question

What is the effect of differences in aliphatic glucosinolate concentrations in *A. thaliana* on herbivores of different feeding guilds and different levels of specialization?

Study-system

To test this, we used several wild-type lines and one transgenic line, that over-expresses aliphatic glucosinolates, of *A. thaliana* . The insects used in this study are two specialists, the leaf chewer *P. rapae and* the phloem sucking aphid *B. brassicae*. For the generalist we used the leaf chewer *S. exigua*.

The MYB28 gene

MYB28 (also referred to as High Aliphatic Glucosinolate 1, HAG1) plays a key role in the synthesis of aliphatic glucosinolates with methionine as the R group. Gigolashvili *et al.* (2007) engineered various *A. thaliana* genotypes with the MYB28 gene. They showed that MYB28-overexpressing genotypes had two to seven fold higher short chain aliphatic glucosinolate concentrations, whereas the indole glucosinolate levels stayed almost unaffected and were only affected when the level of aliphatic glucosinolates expressed was very high, probably because both indole and aliphatic glucosinolates need the same electron source.

When the *A. thaliana* genotypes were damaged by the herbivore *S. exigua*, a chewing generalist, the expression of the MYB28 gene increased very fast for both the wild-type and transgenic genotypes. When comparing the weight gain from the *S. exigua* larvae, the larvae fed with the transgenic MYB28 genotypes had significantly less weight gain (Gigolashvili *et al.* 2007).

Biology of *Arabidopsis thaliana*

Arabidopsis thaliana (L.), thale cress or mouse-ear cress, belongs to the mustard family, Brassicaceae or Cruciferae. *Arabidopsis thaliana* has a wide distribution in Europe, Asia and North America. The plant germinates in autumn and flowers in the winter or early spring, after which it sets seed. Columbia (Col-0) and Landsberg *erecta* are the standard accepted variety of accessions used in research (Meinke 1998). In this research the genotype Col-0, Eringsboda (Eri) and Cape Verde Island (Cvi) are used.

The life cycle of *A. thaliana* is about six weeks. The appearance is small, with flowers of 2 mm in length and rosettes from 2 to 10 cm in diameter. The rosette exists of around 20 leaves which are covered with unicellular trichomes (hairs). The stem can reach a height of 15 to 20 cm when the plant reaches maturity. The roots have no symbiosis with nitrogen-fixing bacteria and their structure is simple. As with almost all plants *A. thaliana* has a wide range of pathogens (Meinke 1998). The herbivores *B. brassicae, P. rapae* and *S. exigua* can feed on this plant (Ratzka *et al.* 2002, Kuśnierczyk *et al.* 2007, Wittstock *et al.* 2004).

Because *A. thaliana* has a relatively short life cycle, small appearance and the whole genome has been sequenced, this plant is ideal for this research.

Research question 1: *What is the difference in morphology and chemistry of the A. thaliana plants?*

Hypothesis 1: Gigolashvili *et al.* (2007) did detect significant morphological changes between Col-0 wild-type and MYB28 genotypes, although it was not studied thoroughly. We also expect morphological differences between the genotypes. The transgenic *A. thaliana* genotypes are expected to express more aliphatic glucosinolates that the wild-type Col-0. Therefore more energy will be used for glucosinolates, which is expected to have a negative influence on the plant growth rate.

Trichome density is expected to be different between the wild-type plants. Between the Col-0 and Col-0 MYB28 plants no difference in trichome density is expected as the inserted gene should have no influence on trichome growth.

How Brevicoryne brassicae copes with glucosinolates

The cabbage aphid *B. brassicae* L. (Hemiptera, Aphididae) is a specialist herbivore that feeds on the phloem sap of brassicaceous plants like *A. thaliana*.

Brevicoryne brassicae can mostly be found on the stem and on young leaves. These parts have a higher glucosinolate level compared to other parts. In spite of the potential negative effects that the higher level of glucosinolates can have on this aphid species, specialists that can cope with the higher glucosinolate concentrations are assumed to feed on these parts to avoid competition and obtain the nutritional better food (Hopkins *et al.* 1998). In their research Gabrys and Tjallingii (2002) also found that *B. brassicae* seems to be using glucosinolates as feeding stimulus.

As a result of the intercellular pathway of aphid stylets towards the phloem when aphids feed on a plant, the plant parts containing myrosinase are not damaged (Tjallingii and Hogen Esch 1993). As a result, there is no myrosinase release and no glucosinolate hydrolysis occurs. When aliphatic or aromatic glucosinolates are not hydrolyzed they form no toxic products that can harm the aphid, therefore performing no negative effect. Indole glucosinolates, on the other hand, do not need myrosinase to hydrolyze them as they are unstable on their own. Indole glucosinolates therefore do have a negative effect on aphids (Kim & Jander 2007, Hopkins *et al.* 2009).

Brevicoryne brassicae sequesters glucosinolates from the host plant and uses these for its own defense against natural enemies (Kazana *et al.* 2007). The aphid mainly sequesters aliphatic glucosinolates, and not indole glucosinolates, probably due to the higher efficiency of aliphatic glucosinolates for defense against its own natural enemies and the toxic effects that indole glucosinolates might have on the aphid itself (Kos *et al.* 2010).

An aphid-specific myrosinase is produced by the aphid and is stored in the nonflight muscle. Because of this, the concentration of this myrosinase is higher in wingless aphids compared to winged aphids. The myrosinase is able to hydrolyze a number of plant glucosinolates like glucotropaeolin and sinigrin into toxic products (Kazana *et al.* 2007). When the aphid gets damaged, the glucosinolates from the plant that are stored in the aphid's body combine with the myrosinase produced by the aphid and as a result, the glucosinolates are hydrolyzed and toxic chemicals are produced. These toxic chemicals have a negative effect on the aphids' predator. Also the chemicals isothiocyanates and nitriles are formed which can act as alarm pheromones to alarm other aphids (Halkier & Gershenzon 2008).

As proof of the negative effect of glucosinolate sequestration by *B. brassicae* on its natural enemies, an experiment by Kazana *et al.* (2007) was done where *B. brassicae* were fed with a diet of 0% and 1% sinigrin. Both the winged and wingless aphids were then fed to larva of the lady bug, *Adalia bipunctata.* The survival of the lady bug was measured. When lady bug larvae were fed aphids from the 0% sinigrin diet the survival rate was 100% for both the winged and wingless aphids. The survival was also high (83.3%) when the lady bug larvae were fed winged aphids from the 1% sinigrin diet. None of the lady bug larvae that were fed wingless aphids from the 1% sinigrin diet survived. This established that the glucosinolate sinigrin plays a key role in the defense of the aphid *B. brassicae* against the lady bug *A. bipunctata*.

Research question 2: *What is the effect of differences in aliphatic glucosinolate concentrations on B. brassicae?*
Hypothesis 2: By feeding on phloem, the aphid only ingests glucosinolates, without the myrosinase enzyme. Aliphatic glucosinolates are therefore not hydrolyzed and the aphid can store them in its body (Kazana *et al.* 2007). Consequently no significant effect of difference in aliphatic glucosinolate concentrations on the performance of *B. brassicae* is expected. But an increase in glucosinolates sequestered within the aphids body is expected as the overall concentration of glucosinolates within the plant is expected to be higher.

How *Pieris rapae* copes with glucosinolates

The small cabbage white*, P. rapae* L. (Lepidoptera, Pieridae), is a specialist on glucosinolate-producing plants like *A. thaliana*. *Pieris rapae* is a leaf chewer, meaning that glucosinolates and myrosinase get mixed when the herbivore is feeding on glucosinolate-producing plants.

Pieris rapae has evolved a way to handle the toxic products that the hydrolysis of aliphatic glucosinolates gives. In the midgut of the larvae a nitrile-specifier protein is produced (Wittstock *et al.* 2004). When aliphatic glucosinolates are hydrolyzed by myrosinase and this nitrile-specifier protein is present, more nitriles are formed instead of isothiocyanates, sometimes even no isothiocyanates at all are formed (Wittstock *et al.* 2004). The higher concentrations of nitriles are less toxic to *P. rapae* then the lower concentration of isothiocyanates.

To test this, Gols *et al.* 2008 used different *Brassica oleracea* L. genotypes found in nature about 10 km apart. When they compared the glucosinolates levels, they found a difference in both aliphatic and indole glucosinolates. *Pieris rapae* was reared on all lines and the development time and pupal mass was measured. In the lines with higher aliphatic glucosinolates levels, no significant difference was found in both development and pupal

mass. In the lines with increased indole glucosinolates on the other hand, both pupal mass and development were influenced, were pupal mass was lower and development was slower.

Müller *et al.* (2010) tested the effects of the absence of indole glucosinolates. When *A. thaliana* plants containing lower concentrations of indole glucosinolates were used to feed *P. rapae*, development was not influenced, but pupal weight was significantly higher.

The reason that indole glucosinolates seem to have a negative effect on *P. rapae* is that these glucosinolates do not need myrosinase to hydrolyze. Therefore the nitrile-specifier protein cannot prevent the formation of isothiocyanates. In an experiment by Agrawal & Kurashige (2003) the effect of increased isothiocyanates was measured on *P. rapae.* Development and mortality was positively affected by increased isothiocyanates concentrations, meaning that the caterpillars grew slower and died faster. Isothiocyanates did not however, have any effect on the pupal mass of *P. rapae.*

Glucosinolates, which *P. rapae* use for recognition of host plants, also seems to have an addictive characteristic on *P. rapae* (Renwick & Lopez 1999). When *P. rapae* larvae were fed cabbage (which contains glucosinolates) before they were fed cowpea (which does not contain glucosinolates), the larvae rejected the cowpea. When the cowpea was treated with extracts from cabbage, the larvae accepted the cowpea as food. Different "start" plants were used, but cowpea was only accepted after treatment with extracts from the first plant.

Müller *et al.* (2010) also tested the effects of the absence of aliphatic glucosinolates. When *P. rapae* fed on *A. thaliana* plants containing lower concentrations of aliphatic glucosinolates, the pupal mass was not influenced, development on the other hand was significantly slower. This could be because there were lower glucosinolates concentrations which are addictive too *P. rapae.*

Research question 3: *What is the effect of differences in aliphatic glucosinolate concentrations on P. rapae?*

Hypothesis 3: For *P. rapae* a significant effect on the performance is expected, as the aliphatic glucosinolates will be hydrolyzed by the plant myrosinase upon feeding. In the presents of the caterpillar's nitrile-specifier protein, the products (nitriles) still possess toxicity, although these are less toxic than when the glucosinolates were hydrolyzed by the plant's enzymes alone (Wittstock *et al.* 2004). Furthermore, the performance of this species might be lower due to the higher investment in detoxification when feeding on plants with higher glucosinolate concentrations.

How *Spodoptera exigua* copes with glucosinolates

The beet armyworm, *S. exigua* Hübner (Lepidoptera, Noctuidae), is a generalist leaf chewer that has a wide variety of host plants. It does, however, not perform equal on each host plant. In a study where the development of *S. exigua* on different host plants was measured there was a significant effect of host plant on development. Azidah & Sofian-Azirun (2006) found a range of 13 days (host plant was long bean) to 27 days (host plant was lady's finger and shallot).

Glucosinolate-producing plants like *A. thaliana* are among the herbivore's hosts and when *A. thaliana* is consumed, glucosinolates and myrosinase mix. In an experiment of Arany *et al*. (2008), *A. thaliana* plants found on different locations were compared by glucosinolates analysis. There was no significant difference in indole glucosinolates

concentrations, on the other hand there was a significant difference in the concentration of aliphatic glucosinolates found between the plants from the dunes and from the inland. The plants from the dunes had a higher concentration of aliphatic glucosinolates. When Arany *et al*. (2008) looked at the weight difference between *P. rapae*, no significant difference in weight was found. *Spodoptera exigua*, however, showed a significant lower weight when reared on the dune plants, containing more aliphatic glucosinolates. It cannot be guaranteed that this was because of the aliphatic glucosinolates, because morphological differences like trichome density were not taken into account.

To determine if glucosinolates increase resistance against *S. exigua* Gigolashvili *et al.* (2007) produced *A. thaliana* lines that over-expressed aliphatic glucosinolates. *Spodoptera exigua* was reared on these transgenic lines and as a control wild-type were used. Within 5 days the fresh weight of the larvae was determined and it was found that the *S. exigua* reared on the glucosinolates over-expressing lines had a 70% lower fresh weight then the larvae reared on the control.

Müller *et al.* (2010) produced mutant *A. thaliana* plants, but instead of overproducing glucosinolates, the mutant lines did not produce (or produced significantly less) indole, aliphatic, or both of these glucosinolates. *Spodoptera exigua* was reared on the wild-type and on all the mutant lines. The dry weight of the larvae was compared and larvae of all the mutant lines were significantly heavier, confirming that glucosinolates act as a plant resistance against *S. exigua.*

Research question 4: What is the *effect of differences in aliphatic glucosinolate concentrations on S. exigua?*

Hypothesis 4: A significantly large effect is expected of *S. exigua* performance when reared on plants with increased concentrations of aliphatic glucosinolates. *Spodoptera exigua* does not have any way of bypassing the toxic effects of glucosinolates, which will hydrolyze inside the body as the caterpillar is a chewing insect. Slower development, lower body-mass and lower survival is expected for *S. exigua.*

Material and methods

Growing of *Arabidopsis thaliana*

The accessions Col-0, Eri and Cvi were used as wild-types. Col-0 was also used to produce the transgenic Col-0 MYB28. This transgenic genotype was produced by Benyamin Houshyani Hassanzadeh at Plant Physiology, Wageningen University by using the *Agrobacterium tumefaciens* vacuum infiltration method described in a paper by Clough & Bent (1998). Using this method the MYB28-gene together with the CaMV 35S promoter and a kanamycin-resistance gene were inserted. The promoter CaMV 35S (Cauliflower mosaic virus promoter) was used to ensure the transcription of the MYB28 gene. This promoter is widely used in plant transformation as it ensures the over-expression of the gene it promotes during all life stages. (Odell *et al.* 1985). Together with the MYB28 gene and CaMV 35S promoter, also a kanamycin-resistance gene was inserted. This was done to give the plant resistance to the antibiotic kanamycin which was important for the selection of transgenic plants.

The seeds from the modified and wild-type plants were sterilized by putting them in open Eppendorftubes that were put in a desiccator filled with a 100ml bleach and 3ml HCL solution overnight. The next day, the transgenic seeds were transferred to a petridish with 50 ml agar-medium containing Murashige and Skoog (MS) medium with vitamins and the antibiotic kanamycin (concentration 30 mg/l) to select for only transgenic seeds. The wild-type seeds were transferred to the same agar-medium, but without kanamycin. After 4 days in dark conditions with a temperature of 4°C the petridishes with seeds were put in a controlled environment with temperature of 21°C ± 2°C, humidity of 60% and a 8/16h photoperiod with a light intensity of 150-200 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). After germination two-week-old seedlings were individually transferred to pots (with a height of 5.5 cm and a diameter of 6.8 cm) with sandy Arabidopsis soil from Lentse potgrond BV (Lent, The Netherlands) sterilized by heating to 80 C for a 4 hour period and kept in the same controlled conditions. Plants were watered three times a week and given entomopathogenic nematodes (Steinernema feltiae; Koppert BV, Berkel en Rodenrijs, The Netherlands) once a week to avoid infestation by sciarid flies. The plants used in all experiments were six weeks old and in a vegetative state.

Chemistry and morphology of *Arabidopsis thaliana*

For the trichomes density, six-week old *A. thaliana* plants were used. Before counting the trichomes, the diameter and number of leaves was determined, only counting the leaves larger then 2mm. The above ground fresh biomass of the plant was measured after trichomes density.

Trichomes were counted in a 0.5 cm x 0.5 cm square which was placed on the middle of the leave, with the main vein in the middle. The trichome density on small, medium and large leaves was measured. Small leaves were just big enough to cover the square. Medium leaves were the 6^{th} or 7^{th} leave counted from the first leave bigger then 2mm. Large leaves were the largest leaves present, not lying on the soil. Of each genotype 10 plants where measured, one leave of each size each.

Glucosinolates were extracted from the plants by using the methods of Van Dam et al. (2004) and Van Dam and Oomen (2008). The HPLC analysis as described by Kabouw et al. (2010) was used. The extraction and analysis of glucosinolates was done by Patrick Kabouw at the NIOO, Heteren.

Brevicoryne brassicae performance

Brevicoryne brassicae was reared on Brussels sprouts (*B. oleracea* L. gemmifera cv Cyrus) under a controlled environment of 16/8h photoperiod, 21°C ± 2°C and a humidity of 60%. Wingless adults were transferred onto six plants of each *A. thaliana* genotype and left overnight to larviposit. Adults were removed the next day. The plants containing the newborn nymphs were transferred to a controlled environment with 8/16h photoperiod, temperature of 21°C ± 2°C and humidity of 60%, and the newborn nymphs were allowed to develop on the plants for 3 days to reach the second instar. At that moment, 20 plants of each genotype were infested with three nymphs that developed on the respective genotype. The development time until first reproduction and the fresh weight of the adult that reproduced the fastest on each plant was measured, and the two slowest developing nymphs were removed from the plants. The number of offspring produced by the fastest

developing adult was counted after a number of days that was equal to the development time until reproduction. Based on these results, the intrinsic growth rate for each of the aphids was calculated. The formula used was intrinsic growth rate = 0.74 x ln(Fd/d), where Fd is the number of offspring produced by an adult aphid during a time period equal to the development time, d (see Karley *et al*. 2002).

To correlate aphid performance with plant chemistry, we collected phloem of aphid-infested plants immediately after the performance experiment ended. For the collection of phloem, the same process as described by Bezemer *et al.* (2005) was used with some minor changes (according to Kos *et al.* 2010). From each aphid-infested plant four fully-grown leaves were collected and put separately into Eppendorftubes containing 200 µl of 8mM EDTA (ethylenediaminetetraacetic acid) for 4 hours, after being put initially in a different EDTA solution for 5 min. to remove any chemicals from the wound resulting from detaching the leaf from the plant. The phloem collected from eight leaves of two plants from the same genotype were pooled and each Eppendorftubes was rinsed with 50 µl EDTA, resulting in ten phloem samples of 2 ml EDTA per genotype.

For the extraction of glucosinolates from phloem a modification of the methods of Van Dam *et al.* (2004) and Van Dam and Oomen (2008) was used. 1 ml (from the 2ml EDTA sample obtained above) was taken and myrosinase was made inactive by boiling the solution in 70 °C and subjecting it to an ultrasonic bath for 15 minutes. After this it was moved onto a Sephadex column. The freeze-dried eluate was resuspended in 100 µl water. The HPLC analysis as described by Kabouw *et al.* (2010) was used.

50 µl of the EDTA samples was used to measure the sugar and amino acid content of the phloem by using the method of Dam and Oomen (2008). This was done for the accessions Col-0, Cvi and Eri.

The extraction and analysis of amino acids and sugars was done by Patrick Kabouw at the NIOO, Heteren.

To test whether aphids that were feeding on plants that contained higher glucosinolate concentrations also contained higher glucosinolate concentrations, we sampled all aphids after the performance experiment ended. Before the aphids were grounded to a fine powder, they were freeze-dried and weighed. For the glucosinolate analysis a total of 50 to 100 mg aphid was collected per plant genotype (Kos *et al.* 2010). Glucosinolates were extracted and analyzed as described above for the plants.

Pieris rapae performance

Pieris rapae was reared on Brussels sprouts under a controlled environment of 16/8h photoperiod, 21°C ± 2°C and a humidity of 60%. Adults were given a Brussels sprouts plant and were allowed to oviposit on the plants for 24h. After 24h the plant was transferred to a controlled environment with 8/16h photoperiod, temperature of 21°C ± 2°C and humidity of 60%. The eggs were allowed to hatch, and neonate larvae were used to infest 30 plants of each accession, Col-0, Cvi and Eri, with one larva each. The larvae were allowed to develop on the plant and if necessary, larvae were given additional plants of the same genotype to sustain survival until the pupal stage. Survival, egg-to-adult development, adult weight and sex of the adult were determined. This experiment was repeated with only using the Col-0 and Col-0 MYB28 genotypes.

To correlate *P. rapae* performance with glucosinolate concentration of the plants they fed on, we infested 10 plants of each genotype with 1 neonate larvae. After 5 days of feeding by the larvae, plants were harvested, freeze-dried, weighed and grounded to a fine powder. Glucosinolates were extracted and analyzed as described above for the plants.

Spodoptera exigua performance

Spodoptera exigua was reared on an artificial diet (see Appendix I) under a controlled environment of 16/8h photoperiod, 27°C ± 2°C and a humidity of 50%. Eggs were allowed to hatch on Brussels sprouts and 25 plants of each genotype where infested with 2 neonate larvae. The plants where put in a controlled environment with 8/16h photoperiod, temperature of 21°C ± 2°C and humidity of 60%. After 9 days the number of larvae on each plant was checked and set to 1 (by removing one larva if both larvae survived; by doing nothing when only one larva survived). The larvae were allowed to develop on the plant and if necessary, larvae were given additional plants of the same genotype to sustain survival until the pupal stage. Survival of the two newborn larvae after 5 days, survival till adult stage, egg-to-adult development, adult weight and sex of the adult were determined.

To correlate *S. exigua* performance with glucosinolate concentration of the plants they fed on, we infested 10 plants of each genotype with 1 neonate larvae. After 5 days of feeding by the larvae, plants were harvested, freeze-dried, weighed and grounded to a fine powder. Glucosinolates were extracted and analyzed as described above for the plants.

Statistical analysis

Analyses were performed using SPSS 17.0 for Windows. Data was analyzed by one-way analysis of variance (ANOVA) and was tested for homogeneity by using the Levene's test. When normality ($P<0.05$) was not reached and transformation failed, a Kruskal-Wallis tests was used. A post hoc Tukey test was done to test for differences between genotypes. Sex ratio was taken into account when analyzes were done of the results form *P. rapae* and *S. exigua*. Survival and sex ratio were tested with Gen Stat 12 using a logistic regression. Differences in means were tested by calculating two-sided t-probabilities.

Results

***Arabidopsis thaliana* characteristics differed among genotypes.** Biomass was found to be different among the genotypes (ANOVA, $F_{3,36}=7.80$; $P<0.001$): Eri plants were heaviest (Table 1). Plants of the genotype Cvi had the largest diameter (ANOVA, $F_{3,36}=7.80$; $P<0.001$) (Table 1). Also leaf number differed among the genotypes (ANOVA, $F_{3,36}=71.17$; $P<0.001$): with Cvi plants having the fewest leaves and Eri plants having the most leaves (Table 1). Trichome density differed for all leaf sizes (small; (ANOVA, F3,36=75.26; $P<0.001$), medium; (ANOVA, F3,36=67.24; $P<0.001$), large (ANOVA, F3,36=63.03; $P<0.001$)) with Cvi plants having the highest trichome density, Eri plants having the lowest density and Col-0 plants together with Col-0 MYB28 plants in between, not being significant different form each other (Table 1). See appendix II for an image of the morphology of the different 6-weeks old *A. thaliana* plants.

Table 1 Different *Arabidopsis thaliana* characteristics (mean ± SE)

Genotype	Col-0	Col-0 MYB28	Cvi	Eri
Biomass (mg)	549.5 ± 27.78 **a**	635.4 ± 47.10 **a**	642.4 ± 32.61 **a**	790.5 ± 32.90 **b**
Diameter (cm)	8.57 ± 0.21 **ab**	8.09 ± 0.17 **a**	10.55 ± 0.23 **c**	9.20 ± 0.19 **b**
Leaf number (#)	23.0 ± 0.39 **a**	26.6 ± 0.90 **b**	18.8 ± 0.42 **c**	29.9 ± 0.53 **d**
Trichomes small leaf (#)	63.0 ± 5.07 **a**	81.1 ± 5.66 **a**	167.4 ± 11.00 **b**	36.2 ± 2.26 **c**
Trichomes medium leaf (#)	26.3 ± 0.97 **a**	25.8 ± 2.04 **a**	60.7 ± 3.25 **b**	17.5 ± 1.28 **c**
Trichomes large leaf (#)	10.7 ± 0.65 **a**	13.4 ± 0.75 **a**	26.3 ± 1.69 **b**	6.8 ± 0.66 **c**

If the letters following the mean ± SE are the same, there is no significant difference (ANOVA, Tukey test, $P<0.05$) n=10 per genotype

Amino acid concentrations did not differ (ANOVA, $F_{2,12}=2.99$; $P=0.089$) among the three accessions (Col-0, Cvi and Eri). Sugar concentrations did differ (ANOVA, $F_{2,12}=3.96$; $P=0.048$): with Eri plants having the highest and Cvi plants having the lowest concentrations (Table 2). Measurements from the genotype Col-0 MYB28 are missing (see Material and Methods)

Table 2 Differences in amino acid and sugar concentration in *Arabidopsis thaliana* (mean ± SE)

Genotype	Col-0	Cvi	Eri
Amino acid (µmol g^{-1})	762.5 ± 77.2 **a**	616.3 ± 96.2 **a**	483.5 ± 65.9 **a**
Sugars (µmol g^{-1})	109.9 ± 11.1 **ab**	75.5 ± 13.8 **a**	133.4 ± 19.1 **b**

If the letters following the mean ± SE are the same, there is no significant difference (ANOVA, Tukey test, $P<0.05$) n=5

Glucosinolate concentrations in plants, phloem and aphids differed among genotypes. The leaf material with both the highest indole (ANOVA, $F_{3,31}=48.81$; $P<0.001$) and aliphatic (ANOVA, $F_{3,31}=109.80$; $P<0.001$) GLS concentrations were leafs from Cvi plants, whereas Col-0 leafs had the lowest concentration of aliphatic GLS and together with Col-0 MYB28 leafs had the lowest concentration of indole GLS. The aliphatic GLS concentrations of Col-0 MYB28 leafs were higher than Col-0 leafs, but were equal to the aliphatic GLS concentrations of Eri leafs (Fig. 1).

For the phloem samples the Col-0 MYB28 genotype is missing as it still needs to be analyzed. Both the indole and the aliphatic GLS concentrations in the phloem followed the trend of the concentrations in the plant, so the difference among the genotypes was the same, with Cvi plants having the highest concentration and Col-0 genotypes having the lowest concentration (aliphatic; (Kruskal-Wallis, $\chi^2=5.67$, $P=0.059$), indole; (ANOVA, $F_{2,12}=23.93$; $P<0.001$), total; (ANOVA, $F_{2,12}=4.68$; $P=0.031$)), although the difference was sometimes less clear (Fig. 2a).

The concentrations of indole and aliphatic GLS found in *B. brassicae* also followed the trend of concentrations in the plant, with Cvi genotype having the highest and Col-0 genotypes having the lowest concentration (aliphatic; (ANOVA, $F_{2,12}=6.02$; $P=0.015$), indole; (ANOVA, $F_{2,12}=0.196$; $P=0.824$), total; (ANOVA, $F_{2,12}=5.27$; $P=0.023$), but again less clear (Fig. 2b).

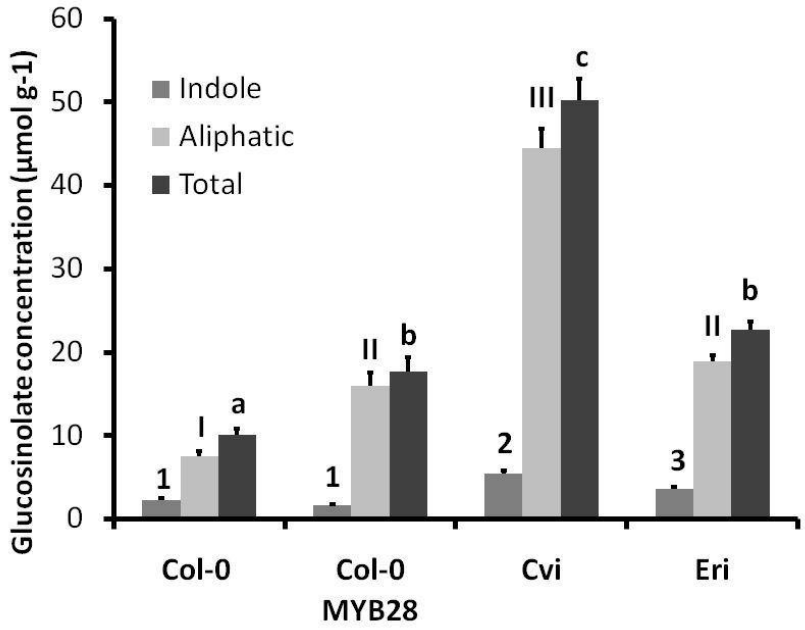

Figure 1. Glucosinolate concentrations (mean ± SE) found in leaf material of the different *A. thaliana* genotypes. If the characters are the same, there is no significant difference (ANOVA, Tukey test, P<0.05), *n*=10, except for Col-0 MYB28 where *n*=5.

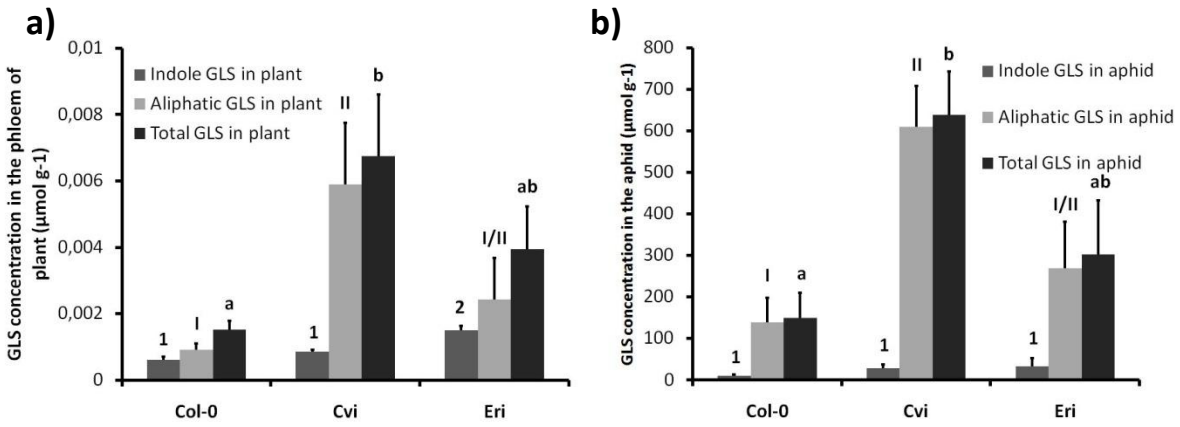

Figure 2. Glucosinolate concentrations (mean ± SE) found in the phloem of the different genotypes (a) and glucosinolate concentrations found in the aphid's body when feeding on the different genotypes (b). If the characters are the same, there is no significant difference (ANOVA, Tukey test, P<0.05), *n*=5.

***Brevicoryne brassicae* performance differed among genotypes.** The intrinsic growth rate of *B. brassicae* was calculated and a significant difference (ANOVA, $F_{3,76}$=27.46; P<0.001) among host genotypes was found, with Cvi plants yielding the highest growth rate and Col-0 plants together with Col-0 MYB28 plants yielding the lowest intrinsic growth rate (Fig. 3).

***Pieris rapae* performance differed among genotypes.** There was a difference in development among the three genotypes (ANOVA, $F_{2,68}$=26.03; P<0.001): development was slowest on Cvi plants and shortest on Eri plants (Fig. 4a). The dry weight of the adult butterfly at the end of the experiment also differed among genotypes (ANOVA, $F_{2,68}$=32.89; P<0.001): *P. rapae* reared on Eri plants was heavier then insects reared on Col-0 and Cvi plants, which were not different (Fig. 4b). At the end of the experiment the survival was also

measured, and survival differed among genotypes (Logistic regression, deviance ratio = 4.40, P = 0.012). *Pieris rapae* caterpillars showed a higher survival on Eri (96.7%) than on Col-0 (70.0%). The survival of caterpillars on Cvi was in between with 80.0% and did not differ from survival on Eri or Cvi. Col-0 MYB28 results are missing because the first experiment was only done with the three wild-types, when the experiment was repeated for Col-0 and Col-0MYB28 genotypes, the survival was dramatically low, around 12%, probably due to a too high humidity in the containers.

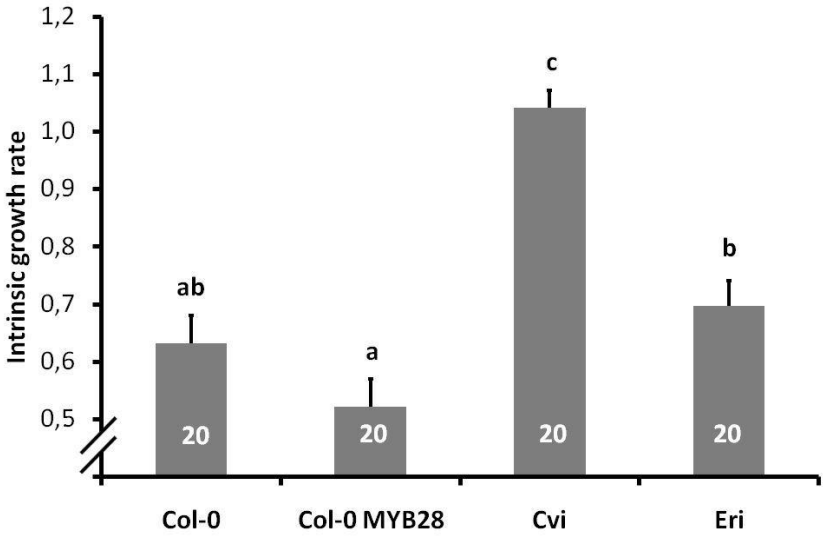

Figure 3. Intrinsic growth rate (mean ± SE) of *B. brassicae* found for the different genotypes. The letters are significantly different (ANOVA, Tukey test, P<0.05), *n* is noted in bars.

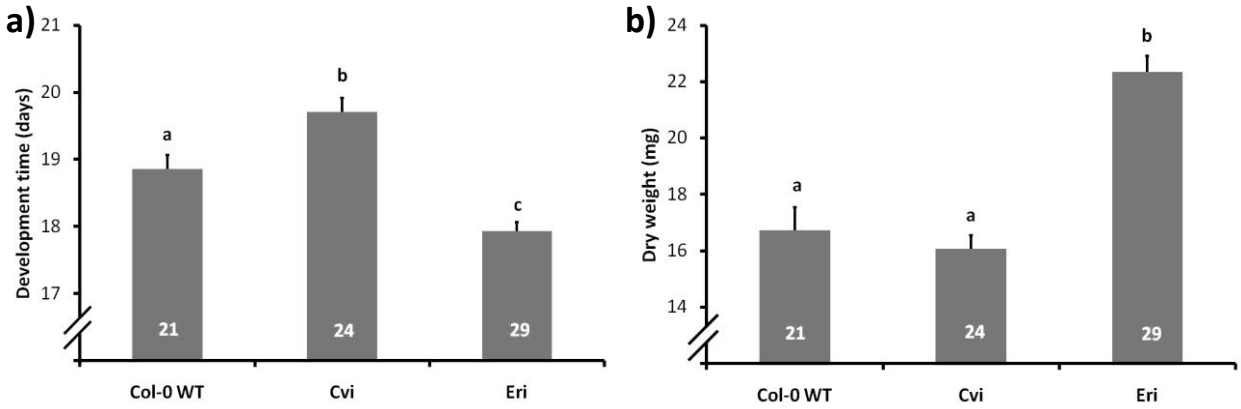

Figure 4. Development time (mean ± SE) (a) and dry weight (mean ± SE) (b) of *P. rapae* reared on the different genotypes. If the letters are the same, there is no significant difference (ANOVA, Tukey test, P<0.05), *n* is noted in bars.

***Spodoptera exigua* performance differed among genotypes.** There was a difference (Kruskal-Wallis, χ^2=19.75, P<0.001) in the development times to pupal stage and total development (Table 3). Both development times were highest for caterpillars reared on Cvi plants and lowest for caterpillars reared on Eri plants. There was, however, no difference (ANOVA, $F_{3,24}$ = 0.96; P=0.427) among the genotypes for the development from pupal till

adult stage (Table 3). Adult dry weight was lower on Cvi plants than on the other genotypes (ANOVA, $F_{3,24} = 10.81$; $P < 0.001$) (Fig. 5b).

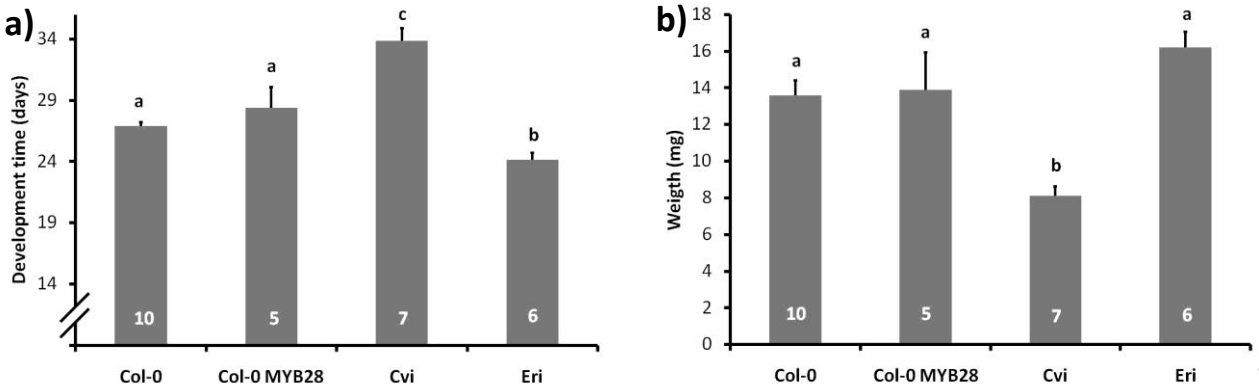

Figure 5. Development time (a) and dry weight (b) of *S. exigua* found for the different genotypes. Values are mean (with SE). If the letters are the same, there is no significant difference (ANOVA, Tukey test, P<0.05), *n* is noted in bars.

Survival was measured in two ways. The survival of the two newborn larvae after 5 days was recorded and the survival of the remaining larva until the adult stage was recorded. For both survivals no difference was found among the different genotypes (survival after 5 days; (Logistic regression, deviance ratio=0.91, P=0.437), survival at the end; (Logistic regression, deviance ratio=1.29, P=0.283)) (Table 3).

Table 3 Survivals and development times of *S. exigua* on different genotypes (mean ± SE)

Genotype	Col-0	Col-0 MYB28	Cvi	Eri
Survival after 5 days (%)	76.7 ± 5.0 **a**	73.3 ± 7.0 **a**	64.7 ± 5.5 **a**	86.0 ± 7.9 **a**
Survival until adult stage (%)	40.0 ± 10.0 **a**	20.0 ± 8.2 **a**	28.0 ± 9.2 **a**	24.0 ± 8.7 **a**
Development time till pupal stage (days)	16.0 ± 0.31 **a**	17.2 ± 0.37 **b**	22.3 ± 0.20 **c**	13.6 ± 0.48 **d**
Development time from pupal to adult stage (days)	10.9 ± 0.31 **a**	11.2 ± 1.53 **a**	11.6 ± 0.92 **a**	10.8 ± 0.38 **a**

If the letters following the mean ± SE are the same, there is no significant difference (ANOVA, Tukey test, P<0.05) and (Kruskal-Wallis. P<0.05) for development time till pupal stage (days)

Discussion

This study was done to test the effect of differences in aliphatic GLS concentrations on the performance of herbivores of different feeding guilds and different levels of specialization. Genotypes differed in chemical and morphological characteristics which led to difference in performance of herbivores of different feeding guilds and different levels of specialization.

***Arabidopsis thaliana* characteristics differences were expected and found among genotypes.** Among the 3 different wild-types almost all morphological and chemical characteristics differed. Eri plants had the highest biomass, although that did not matter in this research, as new plants were provided for the insects when the old plant was eaten. Trichome density differed among the three accessions. Cvi leafs had the highest density of

trichomes, providing this genotype the highest mechanical resistance, whereas Eri leafs had the lowest density of trichomes, providing this genotype the lowest mechanical resistance. Col-0 and Col-0 MYB28 plants had the same density of trichomes. Interesting is that between Col-0 and Col-0 MYB28 only leaf number differed, higher in Col-0 MYB28 plants. We do not have an explanation why the leaf number was higher on transgenic plants. We did, however, expect some sort of phenotypic differences, as was also found by Gigolashvili *et al.* (2007). We do not expect this morphological difference to have had any influence on the results of the herbivore performance experiments. Important is that the trichome density differed greatly among the genotypes, with Cvi having roughly 4 times more trichomes compared to Eri. As we expected, there was no difference in the density of trichomes between Col-0 and Col-0 MYB28 plants.

Total GLS concentrations also differed between the accessions, Cvi plants had the highest, providing this genotype the highest chemical resistance. Total GLS concentrations in Col-0 plants were the lowest, providing this genotype the lowest chemical resistance. Eri and Col-0 MYB28 had the same concentration of total GLS, which is higher than that of Col-0 plants. Between Col-0 and Col-0 MYB28 only the aliphatic GLS concentrations were different, higher in Col-0 MYB28 plants. The aliphatic GLS were expected to be higher as the inserted gene was responsible for overproducing aliphatic GLS.

No effect of higher aliphatic GLS was expected for *Brevicoryne brassicae* performance, but an effect was found. A significant difference in intrinsic growth rate among the genotypes was found. *Brevicoryne brassicae* had the highest intrinsic growth rate on Cvi, which contained the highest concentration of aliphatic GLS. In an experiment done by Hopkins *et al.* (1998) it was also found that *B. brassicae* can be often found on the GLS rich parts of the plant. Most likely this is because of avoiding competition. When looking at our results, it seems that the higher concentrations of GLS may act as a feeding stimulus (Gabrys & Tjallingii 2002). Between Col-0 and Col-0 MYB28 no significant difference in the intrinsic growth rate was found, which in its turn, confirms the hypothesis that aliphatic glucosinolates do not affect performance of *B. brassicae*.

It was expected that there would be a negative effect on intrinsic growth rate from indole GLS in the phloem (Kim & Jander 2007, Hopkins *et al.* 2009). When looking at the indole GLS concentrations in the phloem, it is clear that indole GLS concentrations are equal for Col-0 and Cvi phloem, and highest for Eri phloem. This does not quite follow the indole GLS concentrations in the plants itself, where Cvi is the genotype with the highest GLS concentration followed by Eri, followed by Col-0. But as *B. brassicae* is a phloem feeding insect, the indole GLS concentrations in the phloem of the plant will be used for comparison. Although phloem from Eri has the highest indole GLS concentration, the performance of the aphid on Eri plants is equal to the performance on Col-0 plants, with a lower GLS concentration. It seems that the relative small, but significant, difference in indole GLS concentrations is insignificant by the much larger difference in aliphatic GLS concentrations in the plants phloem.

Negative effect of higher aliphatic GLS was expected for *Pieris rapae* performance. On Eri, the genotype with an average GLS concentration compared to the other genotypes in this study and the lowest density of trichomes, *P. rapae* had the shortest development and the highest weight, resulting in our conclusion that performance was highest on Eri. On Cvi, the

plant with the highest concentration of aliphatic GLS and trichomes, the development was slowest and weight was lowest, both indicating that *P. rapae* performance on this genotype was lowest. Arany *et al*. (2008) found no difference in weight of *P. rapae* among *A. thaliana* plants with different GLS concentrations, and equal indole GLS concentrations. Those findings are dissimilar with the findings presented in this paper. The problem is that not only GLS concentrations, but also trichome density differed among the genotypes used in our study. The next step will be to determine the performance of *P. rapae* on MYB28 genotype plants, where trichome density is unchanged and aliphatic GLS concentrations are increased.

When trichome density and development of *P. rapae* were compared to each other, it was found that the genotype with the highest trichome density resulted in the slowest development and the genotype with the lowest trichome density resulted in the shortest development time. To test this, an entire different angle should be used, not changing the aliphatic GLS concentrations in plants like MYB28 transformation does, but changing the trichome density of plants.

If lower aliphatic GLS concentrations were responsible for better *P. rapae* performance then *P. rapae* would have performed best on Col-0, with the lowest aliphatic concentrations. Instead *P. rapae* had the highest performed on Eri, with average aliphatic GLS concentration. Therefore it could be possible that there is an optimal concentration of aliphatic GLS concentration for *P. rapae*, not too high, for toxicity, and not too low, for plant recognition (Renwick & Lopez 1999).

Pieris rapae is able to by-pass the toxic effect of aliphatic GLS due to a nitrile-specifier protein in its midgut (Wittstock *et al.* 2004). Indole GLS on the other hand are not by-passed with this protein and could therefore still have an effect on the performance of this caterpillar. It appears from our study that indole GLS do not play a large role in the defense against *P. rapae*. Not a clear correlation is found when comparing development time and weight to indole GLS concentrations. Although development time is slowest on the plant with the highest indole GLS, the resulting weight of the caterpillar is equal to that of insects reared on plants with the lowest concentration on indole GLS.

Overall it seems that trichome density is more correlated with *P. rapae* performance than aliphatic GLS concentrations. Differences in aliphatic GLS did have an effect on *P. rapae* performance, but it was unclear whether this was positive or negative, it seems to have an optimum.

Negative effect of higher aliphatic GLS was expected and found for *Spodoptera exigua* performance. As we expected based on previous experiments (Gigolashvili *et al.* 2007, Arany *et al*. 2008) the high aliphatic GLS concentration in Cvi resulted in a slower development and a lower adult weight of *S. exigua.* When reared on Eri the development was shortest and the weight was highest. Like with the results form *P. rapae* it is not clear whether this is due to differences in aliphatic GLS or differences trichome density. When comparing the development and weight of *S. exigua* between Col-0 and Col-0 MYB28 plants, there is no significant difference. As only aliphatic GLS concentrations were different among those genotypes it seems that small differences in aliphatic GLS concentrations do not have any effect on *S. exigua.* But *S. exigua* reared on the genotypes Col-0, Col-0 MYB28 and Eri, with a relative low concentration of aliphatic GLS, had the highest weight. Insects reared on the genotype Cvi, with the highest concentration of aliphatic GLS, yield the lowest weight. Thus aliphatic GLS concentrations do seem to influence the weight of the caterpillar.

When development and trichome density were compared, a correlation seems to appear. *Spodoptera exigua* reared on Cvi, with the highest trichome density, had the slowest development. Insects reared on Eri plants, with the lowest trichome density, had the fastest development. Insects reared on Col-0 and Col-0 MYB28 plants, with a relative average trichome density, had a relative average development.

It seems that for the chewing generalist, trichome density is a negative effect on development and aliphatic GLS concentrations have a negative effect on body weight.

However, the experiment was done with 25 plants for each genotype. At the end only 5 to 10 insects per genotype survived. At the same time, experiments done with *P. rapae* also had much lower survivals than previous, down to only 12%. This is probably due to higher humidity during the experiments. It might therefore be interesting to redo these experiments with reduced humidity.

Effect of differences in aliphatic GLS concentrations and trichomes on different feeding guilds. The main difference between the two feeding guilds is the intake of myrosinase, the enzyme that is responsible for hydrolyzing aliphatic GLS into e.g. isothiocyanates. Phloem feeding insects do not damage the cells where myrosinase is stored, thus do not ingest myrosinase. Leaf chewers on the other hand damage the myrosinase cells and therefore ingest myrosinase.

Comparing the results from the specialist phloem feeder *B. brassicae* with the specialist leaf chewer *P. rapae* a clear distinction can be found for effects of different aliphatic GLS concentrations. The aphid seemed to have a better performance on plants with higher aliphatic GLS concentrations. However, this was not observed between Col-0 and Col-0 MYB28 plants. Although Col-0 MYB28 plants have more aliphatic GLS in their leafs, there is no data showing the aliphatic GLS concentrations in the plant's phloem. Where the phloem feeder seems to do better on higher aliphatic GLS concentrations, the leaf chewer *P. rapae* does not show a clear result in this study, this because there is no data from *P. rapae* feeding on Col-0 and Col-0 MYB28. To get this result, an experiment should be done comparing *P. rapae* performance between Col-0 and Col-0 MYB28 plants. This will exclude the difference in trichome density, which seems to play too big a role in this study.

Effect of differences in aliphatic GLS concentrations and trichomes on different levels of specialization. The main difference between the different levels of specialization, is that when the generalist ingest aliphatic GLS and myrosinase, more isothiocyanates are formed, whereas when the specialist ingest aliphatic GLS and myrosinase, more nitriles are formed.

Comparing the results from the specialist chewer *P. rapae* with the generalist chewer *S. exigua* again shows the problem described above for *P. rapae*. *Spodoptera exigua* on the other hand seems to show more clear effects of differences in aliphatic GLS concentrations. Again trichomes seem to play a large role in *S. exigua* performance, but it seems that a higher aliphatic GLS concentration negatively correlates with weight. Between the Col-0 and Col-0 MYB28 genotype no significant difference was measured, although it was expected. This could be due to the low sample size of insects, or perhaps the aliphatic GLS difference was not large enough. The problem for both these species is that in this study the performance was compared to GLS concentrations inside plants that were not under herbivore attack. From many studies it has become clear that plants can induce GLS when under attack (De Vos & Jander 2009, Textor & Gershenzon 2009, Mewis *et al.* 2005). GLS

concentrations from plants that are under herbivore attack could shed more light on the difference in performance.

Conclusion

It is not clear if plants with increased aliphatic GLS concentrations could be successfully used against insect herbivores in agriculture. *Brevicoryne brassicae* showed higher performance and from other studies it is clear that when introducing a predator, the aphid will have a better defense against its own natural enemies due to accumulated aliphatic GLS, and could thus be more harmful to the crop (Kazana *et al.* 2007). Caterpillars do not accumulate the GLS in their body to be used against predators. But more experiments should be conducted for *P. rapae* to find out whether differences in aliphatic GLS concentrations have an effect on fitness. It seems that an increase in aliphatic GLS has a negative effect on *S. exigua*, making it potential interesting for agriculture.

This study focused on the effect of differences of aliphatic GLS concentrations on herbivore insects only. Even if there would be no significant effect on the herbivore, there could be a significant effect on the predator or parasitoid of that herbivore. Therefore it is very important to expand this research to cover the effects of differences of aliphatic GLS concentrations on the natural enemies of the herbivores.

Beside the potential use of increased aliphatic GLS against herbivores there are two more characteristics of GLS. The bitter taste we experience when eating plants that contain GLS is due to isothiocyanates (Van Doorn *et al.* 1998). This raises the question in what magnitude the taste of the Col-0 MYB28 genotype changes, which is very important for agricultural crops. The other characteristic of GLS that cannot be neglected is its potential for prevention of cancer in mammals (Zhang & Talalay 1994, Wang *et al.* 2010).

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Appendix

I: Artificial diet for *Spodoptera exigua*

5 l water
140 g agar
700 g corn flour
250 g brewer's yeast
250 g wheat germ
10 g sorbic acid
8 g nipagin (methyl, 4 hydroxybenzoate)
40 g ascorbic acid (vitamin C)
0.5 g streptomycin sulphate

II: Morphology of the different 6-weeks old *A. thaliana* plants