Genetics, chemistry and ecology of a qualitative glucosinolate polymorphism in *Barbarea vulgaris*

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Hanneke van Leur

Genetics, chemistry and ecology of a qualitative glucosinolate polymorphism in *Barbarea vulgaris*

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…

En altijd weer de vraag

Of het ooit terecht is
Dat het een gevecht is
Om gewoon ergens te zijn
Om te wonen en te leven
En al was het ook maar even
Echt een mens te kunnen zijn
En dan los te komen van je plaats en tijd

Bløf - Mens

Voor Ron, mijn ouders & Bram

Contents

Summary

Like many other plants, chemical defence compounds are involved in the defense of *Barbarea vulgaris* against natural enemies. *Barbarea vulgaris* produces glucosinolates, which are present in most crucifers such as cabbage, mustard, and the scientific model species *Arabidopsis thaliana*. Glucosinolates form, together with an enzyme (myrosinase), a two-component system: the enzyme and glucosinolates are stored in spatially separated compartments. Upon cell disruption they come into contact, and the glucosinolates are catabolised by the enzyme. For generalist herbivores the glucosinolates, and especially their breakdown products, are often toxic and protect the plants against herbivory. However, specialist herbivores often use these same compounds to recognize suitable host plants. The breakdown products are also responsible for the specific taste to many cabbage and mustard species

There are over 100 different glucosinolates, each with a different chemical structure. The structure determines, amongst other factors, which breakdown product will be formed upon damage. Every plant species has its own typical composition of glucosinolates. I studied how these glucosinolate profiles may affect plant resistance against herbivores.

In a screening of several Dutch populations it was found that *Barbarea vulgaris* plants differed in glucosinolate profile. The majority of the plants contained mainly glucobarbarin, a glucosinolate typical for this species and named after the genus *Barbarea.* Also populations sampled in Germany, Belgium, France, and Switserland, consisted completely of plants with mainly glucobarbarin. In half of the Dutch populations I found that a minority of the plants (2-22%) produced another glucosinolate, named gluconasturtiin. The difference in the chemical structure between these two glucosinolates is very small; glucobarbarin has only one hydroxyl group more than gluconasturtiin. However, this small structural difference may be of large biological relevance. When gluconasturtiin reacts with myrosinase a toxic and unpalatable isothiocyanate is formed, whereas glucobarbarin, due to the position of the hydroxyl group, produces oxazolidinethions. It is unknown whether these oxazolidinethiones are toxic, but in mammals they can inhibit the iodine intake, thereby causing thyroid problems. The *Barbarea vulgaris* glucosinolate polymorphism thus has two chemotypes. I characterized these chemotypes and used them to study the effects of different glucosinolates on herbivores.

The difference in glucosinolate profile is consistently present in all plant organs of *B. vulgaris*, but it is larger in the aboveground organs than in the roots. The glucosinolate profile does not change upon induction by insects nor upon artificial induction by addition of jasmonic acid. I crossed plants, analysed the chemotype of the offspring and showed that the ability to produce glucobarbarin is heritable and regulated by a dominant gene. Based on the assumption that there is a specific enzyme that converts gluconasturtiin to glucobarbarin by a single hydroxylation step, I identified some candidate genes. Further research is needed to determine whether one of these candidate genes is indeed responsible the difference between the chemotypes.

Subsequently, I studied the effect of chemotype on leaf and root herbivores. Plants with mainly glucobarbarin turned out to be very resistant to the generalist leaf-eating larvae of the *Mamestra brassicae* moth. Almost none of the larvae survived on plants with mainly

glucobarbarin. If the larvae were given the choice, they strongly preferred to feed on plants with mainly gluconasturtiin. However, female moths deposited approximately the same number of eggs on each chemotype. Larvae of the specialist small cabbage white grew equally well on each chemotype and did not distinguish between the chemotypes in choice experiments. Larvae of the specialist cabbage root fly, however, performed worse on roots of the gluconasturtiin type than on roots of the glucobarbarin type. Plants responded to the root fly infection by a reduction of the root and shoot biomass with 50%, and decreased levels of nutrients such as sugars and amino acids.

In order to translate the results obtained in the greenhouse to the natural situation, I planted plants of both chemotypes in an experimental garden. Over a period of two years, the numbers of several herbivores were counted on each chemotype every week during the growth season. This revealed that some aboveground insects had a preference for a certain chemotype, and others did not. Butterflies of the small cabbage white preferred to oviposit on plants with gluconasturtiin, but flea beetles and gall midges were more abundant on plants with glucobarbarin. The cabbage aphid and the green peach aphid did not show any preference and were equally abundant on both chemotypes. Three to four times a year, I dug up a subset of the plants to analyse the root herbivores and the soil nematode community. The numbers and community structure of these belowground organisms did not differ between the chemotypes.

Additionally, I investigated whether there are other chemical differences between the chemotypes, apart from glucosinolate profile, that could explain the preference of the above insect species. Therefore, I performed extensive metabolomics analyses, using LC-TOF-MS. Multivariate analyses showed that the major chemical differences between the chemotypes was due to differences in glucosinolates. These differences were larger in shoots than in roots. Apart from glucosinolates only eight unidentified compounds differed between the chemotypes. Known defense compounds such as flavonoids and saponins were identified but did not differ between the chemotypes. Therefore, it is very likely that the differences in herbivore performance and preference are predominantly caused by the differences in glucosinolate profile.

Based on my results, I conclude that the structure of glucosinolates can cause significant differences in resistance against several herbivores. However, in the case of *B. vulgaris* there is no supreme chemotype that is more resistant in all cases. Which of the two chemotypes accrues the largest benefit in a natural environment thus will depend on the herbivore community in their population. In this way the *B. vulgaris* glucosinolate polymorphism can be maintained in natural populations.

CHAPTER 1

General Introduction

Hanneke van Leur

Plant defence and evolution

Plants are susceptible to environmental challenges but cannot run away. Despite this apparent vulnerability the earth's flora has evolved to be highly diverse and abundant (Schoonhoven et al., 1998a). The fact that not all plants are completely consumed can be due to top-down control (Hairston et al., 1960), but also to bottom up mechanisms such as direct defence of plants against herbivores (Ehrlich and Raven, 1964). Plants may play a role in top-down control of herbivores by recruiting natural enemies of their enemies as well: this is called "indirect defence" (Dicke and Sabelis, 1989). Direct plant defence mechanisms can involve morphological adaptations such as trichomes, thorns, or silica bodies in leaves. Additionally, secondary metabolites may be produced, that are toxic or unpalatable to herbivores, or that attract natural enemies of the herbivores (Renwick, 2002). Despite their name, secondary metabolites play a primary role in the chemical interactions between plants and their environment. They are of crucial importance for the attraction of pollinators (e.g. terpenes), protecting the plant against UV light (flavonoids), herbivores and pathogens (e.g. alkaloids, glucosinolates). Most plants contain a high diversity of secondary metabolites (Jones and Firn, 1991). From an evolutionary point this diversity is very intriguing but still poorly understood.

In 1888, Ernst Stahl noted the reciprocal adaptation between plants and their insect herbivores and suggested that chemical compounds may be involved. These early ideas were used by Ehrlich and Raven (1964) to generate a theoretical basis for chemical ecology of insect-plant interactions. They suggested a stepwise chemical coevolution between plants and insects. By chance, a herbivore species evolves tolerance for a plant chemical that is toxic and/or unpalatable to most other herbivores. Eventually the chemical may serve as an attractant and feeding stimulant for a specialist, which has adapted to withstand and even utilize some plant defensive chemicals. This is beneficial for the specialist because the plant represents a niche that is virtually free from competition with other generalist herbivores that are deterred by the chemical (Berenbaum and Zangerl, 1992; Cornell and Hawkins, 2003; Van der Putten, 2003). As a consequence, plants need new compounds to be protected against the specialist (Bak et al., 1999; Nielsen et al., 2001). This process may result in a reciprocal, stepwise "arms race" between insect species and its host plant, leading to a high diversity of chemical defence compounds in plants (Iwao and Rausher, 1997).

Chemical diversity of secondary metabolites

One of the unique features of secondary metabolism is the high number of 'degrees of freedom' of its components. A certain compound can vary qualitatively (structurally) and quantitatively (in concentration) or may even disappear without imminent disastrous consequences for growth and development of the producing organism. Such features are the basis of chemical variation in plants, and consequently the prerequisite for diversification under the selective pressures exerted by a continuously changing environment (Hartmann, 1996).

Chemical diversity in plant compounds is generally derived from differential modification of common backbone structures. This modification requires the evolution of numerous enzymes with different product specificities (Kliebenstein et al., 2001c). Enzyme evolution may start with the substitution, deletion or insertion of a few amino acids, resulting in an alteration of the ratio of products. Another way in which enzymes may evolve, is the insertion of an inverted portion of the complementary sequence, but those insertions are only viable if they occur in non-essential regions of the DNA and when they are not affecting transcriptional elements (Smith, 1997). Sometimes the gene encoding an enzyme duplicates before mutations take place. This gene duplication allows one copy to assume a new function while the other copy maintains its original function, also called "neofunctionalization" (Strickberger, 1995; Pichersky and Gang, 2000; Moore and Purugganan, 2005).

There are various ecological-evolutionary theories aiming to explain the dazzling chemical diversity of secondary plant compounds. First, plants should be able to fight the wide variety of aboveground and belowground herbivores which they may encounter (e.g. herbivorous insects, mites, nematodes and vertebrates, and pathogenic fungi, bacteria and viruses) (Van der Putten et al., 2001; Van Dam et al., 2003; Bezemer and van Dam, 2005). Due to this variation in herbivores and the concomitant range of feeding strategies plant defence requires a broad spectrum of compounds. Having a high diversity of secondary chemicals may provide this broad defence (Rask et al., 2000).

In contrast to a broad-spectrum effectiveness required to fight various herbivores, a highly toxic compound may negatively affect organisms which are directly beneficial for plants (e.g. pollinators) or indirectly (e.g. parasitoids) (Poveda et al., 2003; Soler et al., 2007b). This is more likely when the herbivores belong to the same order as the beneficial organisms and thus share the same physiological features. To avoid this negative side-effect, the plant should be able to discriminate between herbivores and beneficials with similar features and aim its defence at the right organism. This would require a high specificity, and thus a high diversity of chemicals (Simms and Fritz, 1992).

Finally, chemical diversity is driven by the coevolution of herbivores. Because coevolution is most likely to involve only one or a few herbivores species, the other species will remain deterred by the old chemical. As long as a chemical has a function in defence, it may be beneficial to expand the chemical spectrum of a plant, instead of substituting the old chemical with a new one. In brief, the dynamics of plant-herbivore interactions requires also a dynamic and diverse system of chemicals.

Establishment of a new compound: a balance of costs and benefits

When a gene is modified in an individual plant, the fate of this gene depends on how it affects the plant's fitness. A mutation can be deleterious, neutral or advantageous. If mutations are strongly deleterious, they will quickly be eliminated, whereas advantageous ones will soon be fixed in the population by natural selection. When the "new" and "old" gene products are selectively neutral, polymorphisms can become balanced and selection maintains segregating alleles for long periods of time (Mitchell-Olds and Clauss, 2002). Thus

the total balance of costs and benefits in the natural environment of the plant, explains the variation among populations and species in quantity and type of defence. This variation can affect the competition between genotypes and thus the selection for a specific genotype (Simms and Rausher, 1987).

Apart from the obvious benefits described in the previous section, high levels of defence, in the absence of enemy attack, are thought to be costly (Vrieling et al., 1991; Cipollini, 2002). Costs are expressed as the reduction in fitness, or one of its components, for resistant genotypes relative to susceptible genotypes in the absence of herbivores (Bergelson and Purrington, 1996). This prevalence of costs is strongly suggested by the many examples of polymorphisms in defence levels within and among populations (Bergelson and Purrington, 1996).

Costs of defence are often envisioned in terms of allocation of limited resources from other fitness-enhancing functions within a plant, such as photosynthesis, growth and reproduction (Van der Meijden et al., 1988; Herms and Mattson, 1992; Simms, 1992). However, those costs are not always evident, as was calculated for plant volatiles by Dicke and Sabelis (1989) and for terpenoids by Gershenzon (1994). In addition to these allocation costs, chemicals that protect plants against herbivores can be costly to the plant when they are phytotoxic (McKey, 1974; Wittstock and Gershenzon, 2002). Moreover the disruption of an existing biochemical pathway by a new compound may possibly produce costs (Simms, 1992). Some defences may involve ecological trade-offs (Simms and Rausher, 1987). This means, when resources are allocated to defend against one type of herbivore it can reduce the fitness of the plant when damage caused by other herbivores (or a different phenotype of the same species) increases. Finally it is costly when defence compounds deter beneficial organisms such as pollinators and natural enemies of the herbivores (Strauss et al., 1999).

Glucosinolates and the 'mustard oil bomb' defence

Glucosinolates are a well-studied example of a structurally diverse class of defence compounds (Fahey et al., 2001; Mithen, 2001a). They are limited to the order of *Caparales*, which includes the agriculturally important oil seed rape, vegetable crops, such as broccoli and various cabbages, as well as *Arabidopsis thaliana* and *Barbarea vulgaris* (Brown et al., 2003). The limitation of glucosinolate biosynthesis to this single order suggests that it is a comparatively new biosynthetic pathway in the plant kingdom.

Chemically, glucosinolates are amino-acid-derived thioglycosides grouped in classes according to the amino acid from which they are derived: aliphatic/alkenyl glucosinolates are derived from methionine; aromatic glucosinolates are derived from phenylalanine or tyrosine; and indole glucosinolates are derived from tryptophan. The specific side group structures distinguish one glucosinolate from another. To date about 120 glucosinolates have been identified (Fahey et al., 2001). Additional diversity of glucosinolates is due to the secondary modifications of the side-chains by hydroxylations, desaturations and glycosilations after synthesis of the parent glucosinolate (Rask et al., 2000; Graser et al., 2001; Tokuhisa et al., 2004).

The molecular evolution of the glucosinolate pathway probably involved recruitment of genes from other biosynthetic pathways as well as the creation of novel enzymatic activities. The first two stages of glucosinolate biosynthesis, side chain elongation and formation of the core structure, appear to have arisen largely by enzyme recruitment (Graser et al., 2000; Kroymann et al., 2001). In contrast, side chain modification has involved the evolution of new enzyme activities after gene duplication (Kliebenstein et al., 2001b).

Glucosinolates are widely recognized as defensive compounds against herbivores and are involved in host plant recognition by specialist herbivores, thus acting both as an insecticide and an insect feeding attractant (Mithen and Lewis, 1986; Mithen et al., 1987; Rask et al., 2000). Wounding- or grazing-induced cell disruption leads to release of endogenous thioglucosidases (myrosinases) which react with glucosinolates generating a complex array of products e.g. isothiocyanates, nitriles and thiocyanates which in turn have strong effects on herbivores and their natural enemies (Halkier and Du, 1997; Kliebenstein et al., 2001a). Additionally, glucosinolates can have a direct role in plant-plant competition because glucosinolates in root exudates can suppress germination of competitive species, such as grasses (Siemens et al., 2002).

The *Barbarea vulgaris* glucosinolate polymorphism

To assess which molecular-genetic mechanisms and ecological interactions are involved in the evolution of chemical diversity in plants, I used a naturally occurring, qualitative glucosinolate polymorphism in *Barbarea vulgaris* R. Br.. The crucifer *B. vulgaris* (Figure 1.1) is native in Eurasia and is introduced to North America, Africa, and Australia where it is a noxious weed. It grows mainly in grassy vegetation along roads, rivers and ditches. *B. vulgaris* is a biennial or short-lived perennial that forms a rosette in the first year, and produces one or more flowering stalks in the second year (Hegi, 1962). The glucosinolate polymorphism of *B. vulgaris* was first discovered in plants analyzed at the Centre of Terrestrial Ecology (NIOO-KNAW) in Heteren, the Netherlands. In addition to the most common chemotype (BAR-type), whose shoot glucosinolate profile mainly consists of glucobarbarin (*S*-2-OH-phenylethylglucosinolate), up to 22% of the plants in a population may contain gluconasturtiin (2-phenylethylglucosinolate) as the main glucosinolate (NAS-type).

Gluconasturtiin is named after *Nasturtium officinale* and is found in 2 families (Brassicaceae and Resedaceae), in 33 genera and 56 species among which *Arabidopsis thaliana* (Fahey et al., 2001; Reichelt et al., 2002). It is mainly found in root tissues (Sang et al., 1984). Contrastingly, glucobarbarin is only found in 8 species, but these species are still spread over 6 genera and 2 families. This glucosinolate polymorphism was found in seedlings that were grown under uniform conditions in the greenhouse, as well as in flowering plants in natural populations, indicating that this is a genetically determined trait.

Figure 1.1 *Barbarea vulgaris* R.Br., (A) illustrated by R. Westra (Westhof et al., 1971), (B) experimental set-up in greenhouse, (C) in garden experiment, (D) in natural habitat in France.

The difference between the two glucosinolates is only one OH group (see Figure 1.2). The polymorphism is, therefore, thought to be caused by a difference in activity of an enzyme responsible for the hydroxylation of gluconasturtiin. In contrast to *A. thaliana, B. vulgaris* is a poorly studied plant species, although it has additional value for studying ecological aspects of glucosinolates. Because of the longer life span of *B. vulgaris* (Hegi, 1962), the chance to encounter different herbivores is larger than for the ephemeral spring annual *A. thaliana*. Consequently, I expected that more extensive defences are needed for successful establishment of the plant. On the other hand, there is a substantial conservation of gene order for Brassicaceae (synteny) and thus between *A. thaliana* and *B. vulgaris.* Due to this genetic similarity, it is possible to apply the molecular-genetic data from *A. thaliana* to the glucosinolate polymorphism in *B. vulgaris*.

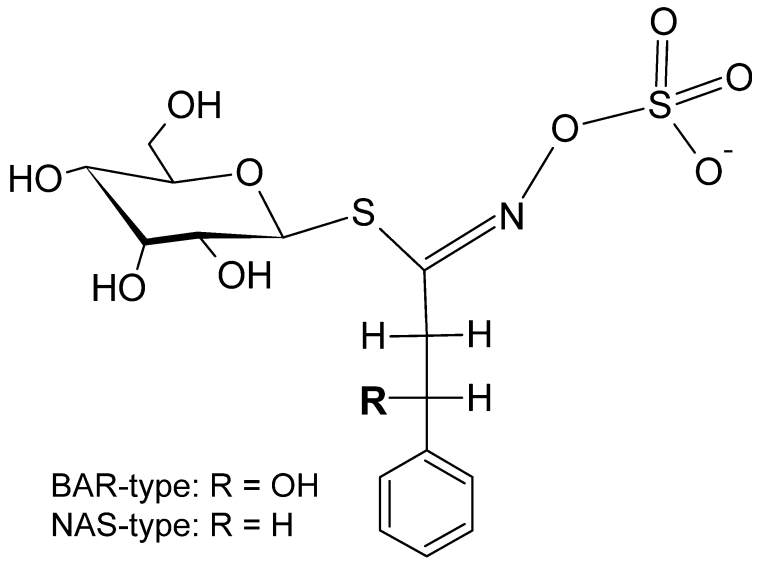


Figure 1.2 Chemical structure of 2-phenylethyl-glucosinolate (gluconasturtiin, R= H) and *S*-2-OH-2-phenylethyl-glucosinolate (glucobarbarin, R = OH).

Glucobarbarin and gluconasturtiin yield different breakdown products. Depending on the presence of cofactors like epithiospecifier protein (ESP), pH or metal ions (Burow et al., 2006), the most likely initial hydrolysis product of glucobarbarin is an isothiocyanate, which due to the 2-hydroxylation of the glucosinolate side chain spontaneously cyclizes to 5-phenyloxazolidine-2-thione (Kjaer and Gmelin, 1957). This glucosinolate breakdown product is known to reduce infection by the soil fungus *Plasmodiophora brassicae* (Ludwig-Müller et al., 1999). Hardly anything is known about other biological activities of oxazolidine-2-thiones (Wittstock et al., 2003).

The most likely breakdown product of gluconasturtiin is 2-phenylethyl isothiocyanate. Isothiocyanates are the predominant breakdown products of glucosinolates and are generally toxic to various herbivores (Wittstock et al., 2003). 2-Phenylethyl isothiocyanate negatively affects a very broad range of herbivores, e.g., nematodes (Potter et al., 1999; Potter et al., 2000; Serra et al., 2002; Lazzeri et al., 2004), snails (Kerfoot et al., 1998), flies, aphids, mites (Lichtenstein et al., 1962), fungi (Sarwar and Kirkegaard, 1998) and several

generalist and specialist Lepidoptera (Wadleigh and Yu, 1988; Borek et al., 1998). Despite counter-adaptations of specialists to reduce or circumvent negative effects of glucosinolates (Ratzka et al., 2002; Wittstock et al., 2003), isothiocyanates can still reduce survival and growth, and increase development time of specialists (Agrawal and Kurashige, 2003). In contrast to the oxazolidine-2-thiones formed in BAR-type plants, which can increase the incidence of goiter in mammals, the 2-phenylethyl isothiocyanate formed in NAS-type plants has chemopreventive effects against tumorigenesis in mammalian organisms (Musk et al., 1995; Griffiths et al., 1998; Canistro et al., 2004a).

The difference in glucosinolates and their breakdown products is likely to cause differences in the ecological interactions of the two *B. vulgaris* chemotypes with herbivores and their natural enemies. This is supported by Rask et al. (2000) who stated that the composition of plant glucosinolate profiles may be more important for mediating plant-insect interactions than the total-glucosinolate concentration. Brassicaceae specialists for example, may be more responsive to particular glucosinolates only present in their preferred host plant. Different ecological interactions may in turn contribute to the emergence and maintenance of this chemical polymorphism.

Metabolomics: going beyond glucosinolates

Heritable defence characteristics can have pleiotropic effects, which may cause additional differences between geno- and phenotypes. To link plant genotypes to the phenotypes in an comprehensive way, we can compare metabolomes (Fiehn, 2002). Although plant metabolomics is widely used and has been found to be applicable to a wide range of disciplines, including the study of gene function, toxicology, plant sciences, environmental analysis, clinical diagnostics, nutrition, and the discrimination of genotypes, there are only few studies in which metabolomics techniques have been applied to plant ecology (Gidman et al., 2003; Kirk et al., 2005; Hendrawati et al., 2006; Liang et al., 2006). Because metabolomic analyses provide information on a large number of metabolites, including their different isotopes, considerable correlations between different signals belonging to one metabolite are expected. The use of multivariate chemometric analyses, such as Principal Component Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA) models, are required to provide an easily interpretable view on the metabolomics information (Jolliffe, 2002; Barker and Rayens, 2003; Rubingh et al., 2006).

For this study, I have chosen to complement our targeted glucosinolate analyses with LC-QTOF-MS analyses (De Vos *et al.*, 2007). LC-QTOF-MS is a widely used metabolomics technique that detects a large group of economically important plant secondary metabolites such as alkaloids, saponins, phenolic acids, phenylpropanoids, flavonoids, glucosinolates, polyamines and derivates thereof. Depending on extraction method and the type of column, also various primary metabolites, such as polar organic acids and amino acids can be reliably analyzed. LC-QTOF-MS is already applied to study taste and defence compounds in tomato and *Arabidopsis thaliana* (Huhman and Sumner, 2002; Tolstikov and Fiehn, 2002; Moco et al., 2006; Rischer et al., 2006).

Herbivores used in this study

Despite the presence of a wide variety of defence compounds, crucifers are still attacked by a broad range of herbivores. In this thesis I compared the reciprocal plant-herbivore interactions between the *B. vulgaris* chemotypes and their AG and BG herbivore communities. I studied aboveground and belowground chemotype-herbivore interactions under controlled conditions in greenhouse experiments and under semi-field conditions in a long term experimental garden experiment.

Aboveground I focussed on common aboveground crucifer herbivores in The Netherlands. I selected the following chewing and sap-sucking generalists and specialists: *Pieris rapae, Mamestra brassicae, Phyllotreta spp, Myzus persicae* and *Brevycoryne brassicae.* Of all these species, the preference or performance is shown to be affected by glucosinolates (Cole, 1997; Rojas, 1999; Nielsen et al., 2001; Wittstock et al., 2003).

The Cabbage root fly, *Delia radicum*, is the main root herbivore described for crucifers and can be a severe pest in natural and in agricultural systems (Finch and Ackley, 1977; Finch, 1993). It is a chewing specialist root herbivore that feeds on the roots of several crucifer species (Block et al., 1987). Shoot glucosinolates are shown to play a role in oviposition host selection of *D. radicum* (Hardman and Ellis, 1978; Roessingh and Städler, 1990; Städler and Schoni, 1990; Roessingh et al., 1992; Hopkins et al., 1997)

To broaden the field study with respect to belowground herbivory and predatory activities, I included analyses of the nematode community. Plant parasitic nematodes can severely reduce plant fitness (Bird and Kaloshian, 2003). The feeding modes of plant parasitic nematodes differ according to the plant parts they feed upon. Ectoparasites feed from the outside of the roots on outer cortical cell layers, while never entering the roots with more than the feeding stylet. These ectoparasites are considered to be feeding generalists (Yeates et al., 1993; Van der Putten et al., 2005). Endoparasitic plant feeders enter the plant root and some of them are specialists (Yeates et al., 1993). Several studies describe a toxic effect of glucosinolate on nematodes (Potter et al., 1999; Buskov et al., 2002; Serra et al., 2002; Lazzeri et al., 2004). Glucosinolate breakdown products potentially affect a broad range of soil organisms, such as bacteria and fungi (Brabban and Edwards, 1995; O'Callaghan et al., 2000; Tierens et al., 2001). Therefore, we also included bacterial feeding, fungal feeding, and omni-carnivore nematodes (Yeates et al., 1993) in our analysis.

Plants are not static victims of herbivores but are known to respond locally and systemically by increasing their defence levels (Bezemer and van Dam, 2005). This is shown for root fly and nematodes infestation (Van Dam et al., 2005) and for many aboveground herbivores (Siemens and Mitchell-Olds, 1996, 1998; Cipollini et al., 2003; Zangerl, 2003; Van Dam et al., 2004; Mewis et al., 2006). To monitor the response of *B. vulgaris* I measured glucosinolate, amino acid and sugar levels upon aboveground and belowground herbivory.

Research objectives and outline of the thesis

In this thesis, I studied a glucosinolate polymorphism of *Barbarea vulgaris.* The main objective was to compare several chemical, physiological and ecological aspects between BAR-type plants and NAS-plants to enhance our understanding of the diversification of secondary metabolites.

In **chapter 2** I started by testing the consistency of the glucosinolate profiles. Therefore, I quantified the glucosinolates in different plant organs, in different developmental stages, and upon induction, using targeted extractions and High Pressure Liquid Chromatography (HPLC) analyses. Subsequently, I selected plants with different chemotypes and performed series of controlled crosses to determine the heritability of the chemotypes. To assess the frequencies of the chemotypes in natural populations, I sampled populations in The Netherlands and surrounding countries. The polymorphism turned out to be heritable, with a dominant gene coding for the ability to produce glucobarbarin. The NAS-type appeared to be under-represented in natural populations. To investigate whether this under-representation of NAS-type plants is due to a relative low fitness of these plants compared to BAR-type plants , I compared the costs and benefits between the chemotypes in chapters 3 to 6.

In **chapter 3** I measured the physiological costs that may be associated with one of the chemotypes. Initially, I focussed on pleiotropic effects, like pathway disruption, which may cause other metabolites to vary consistently between the chemotypes. Therefore, I compared the metabolome of BAR-type and NAS-type roots and shoots by complementing the targeted glucosinolate analyses with untargeted LC-QTOF-MS metabolomics analysis. As physiological differences are only considered to be costly when they affect plant growth or development (Bergelson, 1994), I set up a greenhouse experiment and measured a several growth and development parameters.

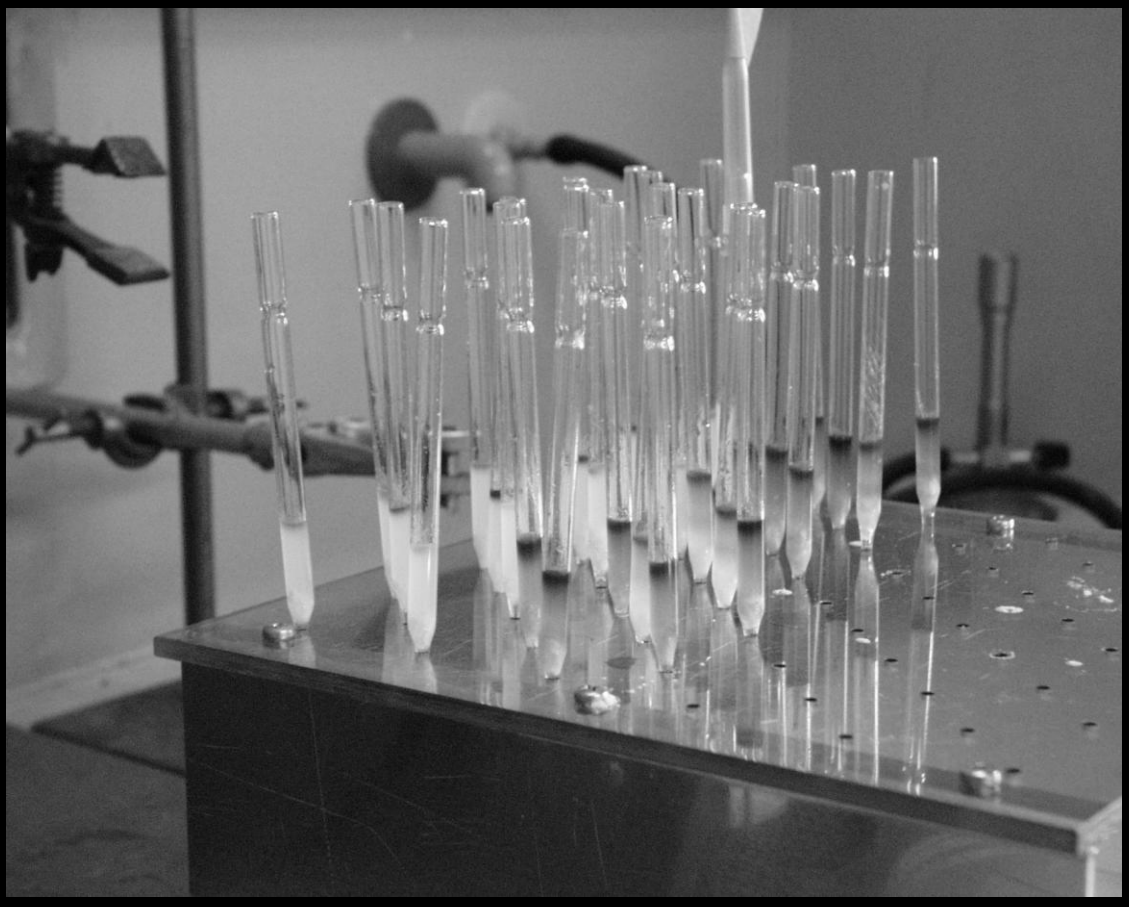
After identification of the costs, I studied the benefits of chemical defence by comparing the performance and preference of several herbivores on BAR-type and NAS-type plants and measuring the damage to the plants. First, I studied interactions between plants and a single herbivore under controlled greenhouse conditions (chapter 4 and 5). In **chapter 4** I tested larval performance and oviposition preference of two aboveground lepidopteran herbivores. I selected the specialist *Pieris rapae* and the generalist *Mamestra brassicae.* To test whether the observed effects could be explained by the nutritional quality of the plant, I also measured plant amino acid and sugar content. In **chapter 5** I tested the effect of chemotype on the performance and oviposition preference of the belowground herbivore *Delia radicum.* Again, I measured the glucosinolate, amino acid and sugar levels of the plants, but in this experiment I also compared these levels between infested and uninfested plants.

Secondly, in **chapter 6** I compared the plant-herbivore interactions between the chemotypes under field conditions. I planted both chemotypes in an experimental garden and censused aboveground and belowground herbivory. As glucosinolates may affect a broad range of organisms, I also analyzed the development of the soil nematode community in time. In the same experimental set up, I investigated plant fitness in the field by measuring biomass, rosette diameter, flowering parameters, and number of seed capsules of both chemotypes when exposed to natural herbivore communities.

Apart from the cost-benefit analyses, I tried to unravel the molecular mechanism behind the polymorphism. In **chapter 7** I discussed which candidate gene could be responsible for the BAR and NAS phenotypes, and described my attempts to identify the gene. Finally, I summarized and discussed the main findings from this thesis and proposed future research directions in **chapter 8**.

CHAPTER 2

A heritable glucosinolate polymorphism within natural populations of *Barbarea vulgaris*



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Abstract

In natural populations of *Barbarea vulgaris* we found two distinctly different glucosinolate profiles. The most common glucosinolate profile is dominated (94%) by the hydroxylated form, (*S*)-2-hydroxy-2-phenylethyl-glucosinolate (glucobarbarin, BAR-type), whereas in the other type 2-phenylethyl-glucosinolate (gluconasturtiin, NAS-type) was most prominent (82%). NAS-type plants have a 108 fold increase of gluconasturtiin concentration in rosette leaves compared to BAR-type plants. The glucosinolate composition of both chemotypes is consistent throughout all plant organs and after induction with jasmonic acid. Although the glucosinolate profile of the roots has a more diverse composition than other plant organs, it still matches the chemotype. In 12 natural populations that we sampled in Germany, Belgium, France and Switzerland solely BAR-type plants were found. However, 8 out of the 15 Dutch populations that were sampled contained 2-22% NAS-type plants. Controlled crosses showed that the chemotype was heritable and determined by a single gene with two alleles. The allele coding for the BAR-type was dominant and the allele for the NAS-type was recessive. The different glucosinolate profiles will yield different hydrolysis products upon damage, and therefore we expect them to differentially affect the multitrophic interactions associated with *B. vulgaris* in their natural environment.

Keywords

Barbarea vulgaris; Brassicaceae*;* Glucosinolates*;* Glucobarbarin; Gluconasturtiin; Plant defence*;* Polymorphism; Heritability.

Introduction

The defence capacity of plants is strongly determined by their secondary metabolites. The profiles of secondary metabolites can differ among species, among populations of the same species and also among individual plants within a population. Even within an individual plant, control mechanisms operating on biosynthesis, transport and catabolism of secondary metabolites can be organ specific (Sang et al., 1984; Zhang et al., 1991; Hartmann, 1996; Brown et al., 2003). All this variability is restricted by the plant's genome (Kliebenstein et al., 2001c; Cipollini et al., 2003) and can be influenced by ontogeny (Boege and Marquis, 2005), induction by other organisms (Agrawal et al., 1999; Dicke et al., 2003; Van Dam et al., 2003) or seasonal changes (Agerbirk et al., 2001a).

A highly diverse and variable group of plant defence compounds are the glucosinolates (reviewed by Halkier and Gershenzon, 2006). A thioglycosylated sulfated oxime is an important structural feature of all of the more than 100 known glucosinolates, which are mainly distinguished by variations in the amino acid derived carbon sceleton known as the 'side chain' (Mithen, 2001b). Upon tissue damage, glucosinolates are hydrolysed by a thioglucosidase, commonly known as the enzyme myrosinase, which results in a range of toxic and noxious compounds, such as isothiocyanates, thiocyanates, nitriles, and oxazolidine-2-thiones. Isothiocyanates are the most toxic among the hydrolysis products, they even affect specialist herbivores (Agrawal and Kurashige, 2003). Nitriles and thiocyanates have a lesser toxicity to insects (Lambrix et al., 2001), whereas hardly anything is known about the biological effects of glucosinolate-derived epithio-nitriles and oxazolidine-2-thiones on insect herbivores (Wittstock et al., 2003). Which of the exact hydrolysis products are formed, depends strongly on the structure of the glucosinolate side chain (Wittstock et al., 2003).

Here we present a glucosinolate polymorphism in *Barbarea vulgaris* which is based on distinct glucosinolate profiles *within* populations. The main difference in glucosinolate profiles between the two chemotypes is the hydroxylation of the side chain of the most abundant glucosinolate. In most *B. vulgaris* plants the main glucosinolate is glucobarbarin, which has a 2-hydroxylated 2-phenylethyl side chain. In several Dutch populations we found that 2-22% of the plants contained the unhydroxylated gluconasturtiin (2-phenylethylglucosinolate)as their main glucosinolate. This minor difference in chemical structure is expected to have major consequences for the biological function. Upon hydrolysis, 2-phenylethylglucosinolate will produce a volatile isothiocyanate, whereas the 2-hydroxylated form may yield an oxazolidine-2-thione (Kjaer and Gmelin, 1957; Wittstock et al., 2003). This difference in hydrolysis products is likely to have very different effects on the multitrophic interactions associated with *B. vulgaris*. In contrast to the *B. vulgaris* polymorphism described by Agerbirk et.al. (2003a), our polymorphism did not include any visible morphological differences. These two aspects make this polymorphism an excellent candidate for testing questions about the molecular-evolution of glucosinolate diversity.

In this paper we show that the differences in glucosinolate profiles between the two types are consistent throughout the plant and after induction with jasmonic acid. Furthermore we describe the geographic distribution of the polymorphism in 27 natural populations in The Netherlands and surrounding European countries. Finally, we present a sequence of crosses to show that the glucosinolate type is heritable.

Results

First we assessed the consistency of the polymorphism by sampling different organs of flowering *B. vulgaris* plants, as well as by analysing leaves of jasmonate induced rosette plants. Second, we censused 27 geographically separated natural *B. vulgaris* populations, in the Netherlands and in surrounding European countries, to asses the frequency of the polymorphism. Finally, we performed a series of controlled crosses to asses the heritability of glucosinolate type in *B. vulgaris*.

Glucosinolate profiles

Although the chemotypes contained the same set of glucosinolates they were clearly distinguished by the quantities of their main glucosinolate (Figure 2.1). We detected three aromatic glucosinolates (glucobarbarin, gluconasturtiin, and glucosibarin) and three indole glucosinolates (glucobrassicin, neoglucobrassicin and 4-methoxyglucobrassicin). BAR-type plants contained mainly glucobarbarin (aboveground organs ca. 94%, roots ca. 38% of all glucosinolates), and the NAS-type contained mainly gluconasturtiin (aboveground organs ca. 81%, roots ca. 62% gluconasturtiin). The prevalence of the main glucosinolate in the profile of the two chemotypes was consistent in all organs (Figure 2.1, pie-charts).

Overall, the total amount of glucosinolates did not differ between the types (ANOVA type-effect: $F_{1,44} = 1.98$, $P > 0.05$; bar-graphs in Figure 2.1). The distribution of the total amount of glucosinolates over the organs was also not different between chemotypes (ANOVA type × organ: $F_{4,44} = 0.74$, $P > 0.05$). Tukey post-hoc tests per organ, however, showed that seeds and flowers had a higher glucosinolate content in BAR-type plants than in NAS-type plants (seeds, $P = 0.003$; flowers, $P = 0.01$). Within plants, the total glucosinolate content significantly differed between organs (ANOVA organ-effect: $F_{4,44} = 5.03$, $P = 0.002$).

All organs showed a highly similar glucosinolate composition except for the roots. Roots of both types had a relatively high neoglucobrassicin content compared to other organs (% of total glucosinolates: roots NAS-type = 7.1%, roots BAR-type = 3.9% all other organs < 0.03%). Another type inconsistency was shown in BAR-type roots, which had a gluconasturtiin content of 36.1%, which was almost equal to the glucobarbarin content (38.3%). This was a major difference compared to the other organs of BAR-type plants that contained on average only 1.5% gluconasturtiin. Despite the higher levels of gluconasturtiin in BAR-type roots, the dominant glucosinolate still matched the type.

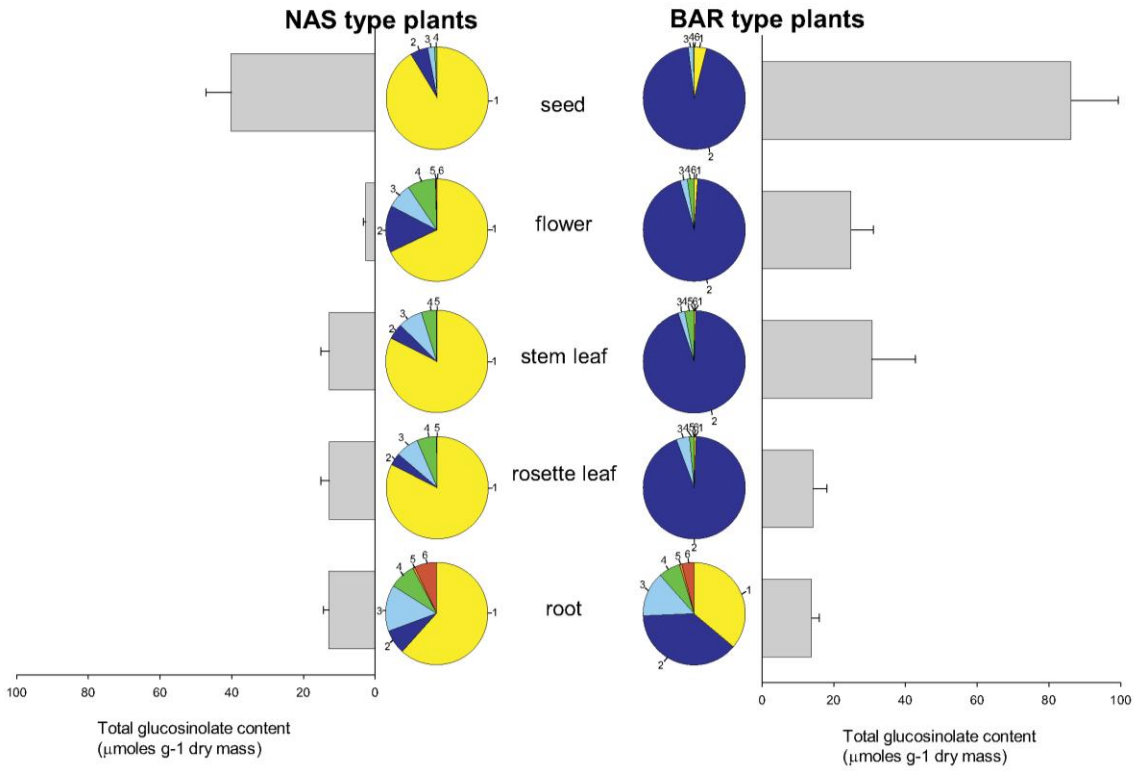

Figure 2.1 The total glucosinolate levels (graphs, µmoles dry mass + SE) and relative abundance of glucosinolates (pie charts, percentage of total glucosinolates) in seeds (n = 18 per type), roots, rosette leaves, stem leaves and flowers of NAS-type plants (left, n = 3) and BAR-type (right, n = 3) of *Barbarea vulgaris* plants.

Number, slice color, common names and systemic names (in parentheses) of the glucosinolates found in *B. vulgaris*: **1**, yellow, gluconasturtiin (2-phenylethylglucosinolate); **2,** dark blue, glucobarbarin ((*S*)-2-hydroxy-2-phenylethylglucosinolate); **3**, light blue, glucosibarin, ((*R*)-2-hydroxy-2-phenylethylglucosinolate); **4**, green, glucobrassicin, (indol-3-ylmethylglucosinolate); **5**, orange, 4-methoxyglucobrassicin, (4-methoxyindol-3-ylmethylglucosinolate); **6**, red, neoglucobrassicin (*N*-methoxyindol-3-ylmethylglucosinolate).

Consistency of the chemotype after induction

Jasmonic acid (JA) treatment significantly altered total glucosinolate levels in both types (MANOVA, JA treatment effect, $F_{10,82} = 2.32$, $P = 0.018$). Protected contrast analysis showed that overall the total glucosinolate level increased after JA application (contrast CON vs. SJA and RJA, t = 2.38, p = 0.02), independently of where the JA was applied (contrast RJA vs. SJA, t = 0.99, p = 0.32). The effect of JA on total glucosinolate level was more pronounced in NAS-type plants than in BAR-type plants (Figure 2.2). Most importantly, JA induction did not change the dominance of the main glucosinolate in either chemotype: the relative contribution of the main peak to the glucosinolate pattern remained similar for all treatment groups within a chemotype (Figure 2.2, percentages in the bars). Induced responses therefore are not likely to affect chemotype classification of plants sampled in natural populations. Interestingly, leaves of NAS-type rosette plants had significantly lower total levels of glucosinolates than those of BAR-type rosettes (Figure 2.2, letters over bars), which is consistent with the lower total glucosinolate levels in reproductive parts of NAS-plants (Figure 2.1).

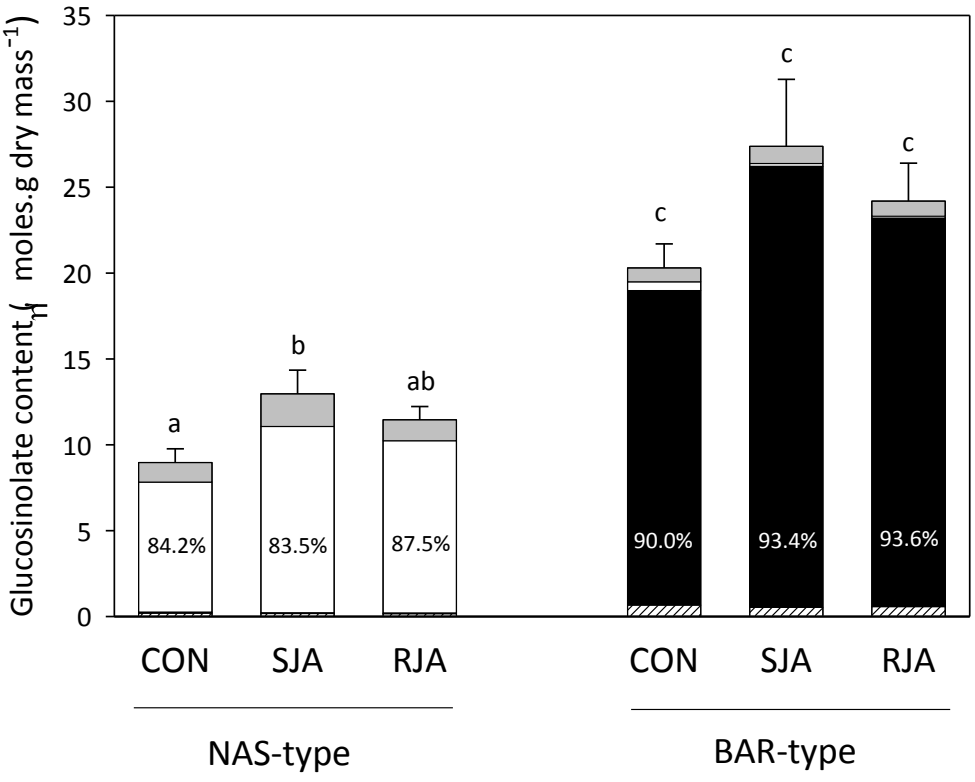

Figure 2.2 Glucosinolate concentration in rosette leaves of *B. vulgaris* NAS or BAR plants treated with 500 µg jasmonic acid (JA) to their shoots (SJA) or to their roots (RJA). Controls (CON) were treated with acid water to control for effects of acid application. Error bars indicate standard error of the mean of total glucosinolate levels. The percentages in the bars indicate the relative contribution of the main peak (NAS or BAR) to total glucosinolate level. n = 8 for all NAS type groups, and n = 8, 9 and 10 for the BAR-type treatment groups, respectively. Hatched bar: glucosibarin; open bar: gluconasturtiin; black bar: glucobarbarin; grey bar: sum of glucobrassicin and 4-methoxyglucobrassicin.

Geographical distribution of both chemotypes

Besides the most common BAR-type, we detected 2-22% of NAS-type plants in eight geographically separated populations in The Netherlands. In the other Dutch populations, in the two commercial seed batches, and in all populations outside The Netherlands no NAS-type plants were detected (Figure 2.3 and Table 2.1). The existence of several mixed populations suggests that NAS-types have established in some populations but did not disperse to or did not become established in all populations. Leaf glucosinolate profiles of BAR- and NAS-type plants were consistent throughout all populations.

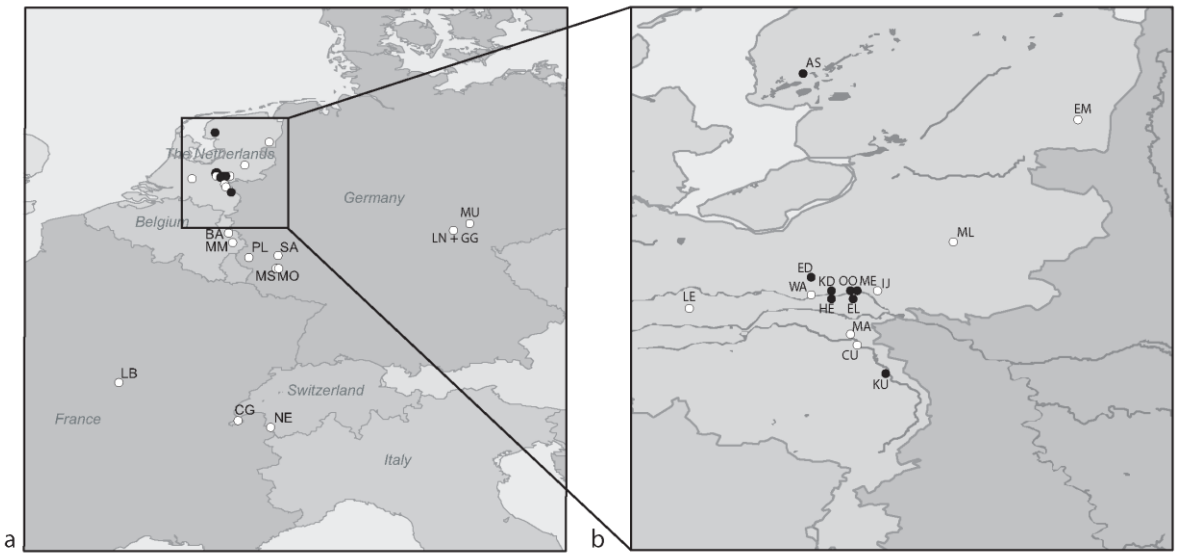

Figure 2.3 Geographical distribution of sampled populations. Black spots are populations in which the chemotypes co-occur. White spots are the populations which contain solely BAR-type plants. Additional information about the populations is given in Table 2.1. A) Overview of all sampled populations. B) Dutch populations only.

Table 2.1 Site descriptions of the 27 natural populations of *B. vulgaris* and the distribution of the chemotypes.

Code	Population name	Country [a]	Long. [b]	Lat. [c]	Pop. size [d]	No. of plants sampled	% BAR chemotypes
MU	Maua	DE	11.60	50.87	small	10	100%
MM	Malmedy	BE	6.03	50.42	medium	49	100%
LN	Leutnitz	DE	11.22	50.70	small	10	100%
GG	Grossgöllitz	DE	11.22	50.70	large	10	100%
BA	Baelen	BE	5.97	50.63	medium	38	100%
SA	Schöne Aussicht	DE	7.12	50.22	small	42	100%
PL	Plütscheid	DE	6.42	50.08	small	23	100%
MS	Merscheid	DE	7.07	49.83	medium	29	100%
MO	Morbach	DE	7.12	49.82	small	22	100%
LB	Lurcy-Le-Bourg	FR	3.38	47.16	small	9	100%
NE	Neuchatel	CH	6.93	46.10	small	11	100%
CG	Creux-de-Genthod	CH	6.17	46.27	large	48	100%
AS	Alde Skatting	NL	5.63	53.00	large	50	94%
EM	Emmen	NL	6.92	52.78	small	20	100%
ML	Markelo-Laren	NL	6.33	52.22	small	16	100%
ED	Ede	NL	5.07	52.05	small	23	96%
KD	Kievitsdel	NL	5.67	51.98	large	50	80%
OO	Oosterbeek	NL	5.85	51.98	large	50	98%
IJ	Ijsseloord, Arnhem	NL	5.98	51.98	large	50	100%
ME	Meinerswijk	NL	5.88	51.98	small	33	97%
WA	Wageningen	NL	5.67	51.97	medium	50	100%
EL	Elderveld, Arnhem	NL	5.87	51.95	large	50	78%
HE	Heteren	NL	5.77	51.95	large	50	96%
LE	Leerdam	NL	5.10	51.90	medium	50	100%
MA	Malden	NL	5.85	51.78	large	50	100%
CU	Cuijk	NL	5.88	51.73	large	50	100%
KU	Kiekuut-Vierlingsbeek	NL	6.02	51.60	medium	50	88%

[a] ISO abbreviations are used for country names.
[b] Long. = longitude (°E)
[c] Lat. = latitude (°N)
[d] Population size small is < 50, medium 51-250, and large is > 250 plants

Heredity

The frequency of the two chemotypes in the half-sib offspring from randomly crossed BAR-type and NAS-type field plants depended on the maternal chemotype. The BAR-type parents yielded on average 82% BAR offspring, whereas NAS-type parents yielded only 28% of BAR-type offspring (G-test of independence, G = 37.14; $P < 0.01$). These results were the first indication that glucosinolate chemotype is a heritable trait. Because some of the offspring had a chemotype different from the mother plant, we concluded that the heritability was not completely maternal.

Binary qualitative traits are often determined by a single gene with one dominant and one recessive allele (e.g. Van Dam et al., 1999). We hypothesized that this also applied to the *B. vulgaris* polymorphism. To test this hypothesis we performed a series of controlled crosses. First we crossed NAS-type half-sibs (F1) from a NAS-type mother (P), with each other. All resulting offspring (F2) was of the NAS-type (Table 2.2, cross 1-10). Because the mothers of these plants were NAS-type as well, the NAS-type F1 plants were supposed to be homozygous, whereas their BAR-type half-sibs were supposed to be heterozygous. Because the NAS-type P plants produced a mixed offspring after random crossing in the field, this also suggested that the allele coding for BAR-type is dominant.

Indeed, crosses of two allegedly heterozygous BAR-type plants consistently showed that the observed ratio of phenotypes in the offspring did not deviate from the expected 1:3 (NAS-type:BAR-type) ratio ($G_{heterogeneity}$ = 2.100, p = 0.717; G_{pooled} = 2.20, *P* = 0.698; G_{total} = 4.3, *P* = 0.366; Table 2.2, cross 11-18). This indicated simple Mendelian inheritance of the glucosinolate type, coded by a single gene, with the BAR-type dominant over the NAS-type. This assumption was confirmed by crossing the same heterozygous BAR-type plants with a homozygous NAS-type plant from the same generation, which yielded a phenotype distribution in the offspring that did not significantly differ from the expected 1:1 ratio (G_{pooled} = 0, *P* = 1 G_{total} = 2.160, *P* = 0.706; Table 2.2, cross 19-26). Additionally, we selected BAR-type plants of which selfings produced a uniform BAR-type offspring (Table 2.2, cross 27-28) suggesting that these plants were homozygous. When placed in our experimental garden, which contained both BAR- and NAS-type plants, these plants indeed yielded only BAR offspring as well (Table 2.2, cross 29-30), confirming our assumption that these two plants were homozygous dominant. Based on these consistent results, we conclude that the chemotypes are heritable and that the allele coding for BAR-type is dominant and the allele coding for the NAS-type is recessive.

Table 2.2 Expected and observed frequencies of both chemotypes in the offspring of controlled crosses. The origin refers to the natural population of the parent as described in Table 2.1 and depicted in and Figure 2.2. In the columns P and F1 the numbers refer to the parent plant and the letters to the half-sib individuals.

Cross	Origin	P	F1♀		F1 ♂	$F2_{expected}$		$F2_{observed}$		No. F2 sampled
		NAS	NAS	×	NAS	%NAS	%BAR	%NAS	%BAR	
1	OO	1	1a		1b	100	0	100	0	25
2	OO	1	1b		1a	100	0	100	0	25
3	EL	1	1a		1b	100	0	100	0	50
4	EL	1	1b		1a	100	0	100	0	50
5	EL	2	2a		2b	100	0	100	0	12
6	EL	2	2b		2a	100	0	100	0	12
7	EL	3	3a		3b	100	0	100	0	25
8	EL	3	3b		3a	100	0	100	0	13
9	EL	3	3b		3c	100	0	100	0	22
10	EL	3	3c		3b	100	0	100	0	28
		NAS	BAR	×	BAR	%NAS	%BAR	%NAS	%BAR	
11	OO	1	1c		1d	25	75	33	67	24
12	OO	1	1d		1c	25	75	33	67	27
13	EL	1	1c		1d	25	75	27	73	49
14	EL	1	1d		1c	25	75	23	77	39
15	EL	2	2c		2d	25	75	44	56	25
16	EL	2	2d		2c	25	75	38	63	24
17	EL	2	2e		2e	25	75	21	79	24
18	EL	3	3d		3d	25	75	27	73	48
		NAS	BAR	×	NAS	%NAS	%BAR	%NAS	%BAR	
19	EL	1 / 3	3d		1a	50	50	no	no	0
20	EL	1 / 3	1c		3a	50	50	56	44	18
21	EL	1 / 3	1d		3b	50	50	no	no	0
22	OO	1	1c		1e	50	50	52	48	25
		NAS	NAS	×	BAR	%NAS	%BAR	%NAS	%BAR	
23	EL	1 / 3	1a		3d	50	50	44	56	25
24	EL	1 / 3	3a		1c	50	50	56	44	16
25	EL	1 / 3	3b		1d	50	50	40	60	25
26	OO	1	1e		1c	50	50	56	44	25
		BAR	BAR	×	BAR	%NAS	%BAR	%NAS	%BAR	
27	HE	1	1a		1a	0	100	0	100	23
28	HE	1	1b		1b	0	100	0	100	21
		BAR	BAR	×	Random	%NAS	%BAR	%NAS	%BAR	
29	HE	1	1a		Random	0	100	0	100	24
30	HE	1	1b		Random	0	100	0	100	25

Discussion

Quality of glucosinolates

The heritable glucosinolate polymorphism in *B. vulgaris* that we found may have several profound ecological implications. Due to the different end products of the glucosinolates, the two chemotypes may differentially affect both root and shoot herbivores that are associated with *B. vulgaris*. In the NAS-type gluconasturtiin was consistently the most abundant glucosinolate, in all tissues and after induction with jasmonic acid. Upon tissue disruption gluconasturtiin reacts with myrosinase, which hydrolyses the thioglucoside bond resulting in an unstable aglycone that spontaneously may rearrange to 2-phenylethyl isothiocyanate (Chew, 1988; Wittstock et al., 2003). The volatile and pungent isothiocyanates may be toxic or deterrent to a broad range of organisms for example fungi (Tierens et al., 2001), nematodes (Potter et al., 1999; Serra et al., 2002), snails (Kerfoot et al., 1998), insect herbivores (Wittstock et al., 2003) and other plants (Yamane et al., 1992). Additionally, isothiocyanates are involved in indirect defence by attracting insect parasitoids and parasites (Wittstock et al., 2003). This defence system is commonly known as the 'mustard oil bomb' (Ratzka et al., 2002). On the other hand, a number of specialist herbivores like *Pieris rapae, Delia radicum*, and *Phyllotreta nemorum* are immune to the 'mustard oil bomb', and have been found to be attracted to the volatile isothiocyanate, as well as using the intact glucosinolates as oviposition cues (Chew, 1988; Nielsen, 1997; Städler et al., 2002). The most abundant glucosinolate of the BAR-type plants, glucobarbarin, may yield 2-hydroxy-2-phenylethyl isothiocyanate, but the presence of the 2-hydroxygroup spatially close to the electrophilic isothiocyanate carbon results in immediate cyclisation to 5-phenyloxazolidine-2-thione (Kjaer and Gmelin, 1957). This product is non-volatile, thus not likely to attract ovipositing insects from a distance, but also non-reactive. Therefore we expect that it does not have the same defence characteristics as an isothiocyanate. On the other hand, oxazolidine-thiones are known to inhibit infection with the soil fungus *Plasmodiophora brassicae* (Ludwig-Müller et al., 1999) and to have strong antinutritional efffects on mammals (Fenwick et al., 1983). This means that also this type of product from the 'mustard oil bomb' may play a role in plant defence, albeit a different one.

Belowground the differences between the types were less pronounced, which may be due to the fact that gluconasturtiin is a typical root glucosinolate; high levels of gluconasturtiin were found in roots of diverse crucifer species whereas their shoots hardly contained gluconasturtiin (Sang et al., 1984; Kirkegaard and Sarwar, 1998; Agerbirk et al., 2003a; Van Dam et al., 2004). This is evident also in the BAR-type, where all organs contain mainly glucobarbarin but the roots have a relatively high gluconasturtiin content. The slightly higher gluconasturtiin level in BAR-type seeds compared to BAR-type leaves and flowers may be explained by the presence of primordial root and shoot tissue in seeds.

Quantity of total glucosinolates

Not only glucosinolate composition, but also total glucosinolate level may be important for plant defence. The total glucosinolate level is reported to affect herbivores in a dose-dependent way (Louda and Mole, 1991; Li et al., 2000; Agrawal and Kurashige, 2003). In rosette plants, total leaf levels were higher in BAR-types. Similarly, seeds and flowers of BAR-type plants had a higher total glucosinolate content than the reproductive organs of NAS-

type plants. Therefore, the rosettes and reproductive organs of flowering BAR-type plants are potentially better protected against herbivores, unless the differences in the glucosinolate composition between the types is a more important determinant of herbivore resistance than total concentration.

Differential distribution of glucosinolates over different organs has been frequently described (Louda and Mole, 1991; Strauss et al., 2004) and it is assumed to reflect optimal defence allocation. Organs that are most closely linked to plant fitness, such as seeds and flowers, are predicted to be defended constitutively at higher levels than leaf tissue (Zangerl and Nitao, 1998). Indeed, the seeds had the highest total glucosinolate content in both types, which is consistent with earlier findings in other crucifer species, such as *Arabidopsis thaliana* (Brown et al., 2003), and *Brassica juncea* (Palmer et al., 1987). Between the types, the anatomical distribution of total glucosinolate contents did not differ.

Genetic background

It has been postulated that heritable secondary metabolite polymorphisms like the one we describe here, are due to random gene duplications (Mitchell-Olds and Clauss, 2002; Ober, 2005). In the case of the *B. vulgaris* glucosinolate polymorphism, however, we hypothesize that the less frequent NAS-type has arisen from a loss-of-function mutation in the most common BAR-type. Loss-of-function mutations are generally recessive (Stacey, 1994; Van Dam and Baldwin, 2003). A similar enzyme, belonging to the 2-oxoglutarate dependent dioxygenase (2-ODD) enzyme family, is reported to convert methylsulfinylalkenyl glucosinolates into hydroxyalkenyl glucosinolates (Kliebenstein et al., 2001b). *Arabidopsis thaliana* ecotypes missing the gene coding for the 2-ODD enzyme, did not contain the hydroxylated glucosinolates. We propose that a similar 2-ODD enzyme converting gluconasturtiin into glucobarbarin is damaged, has a lower expression level, or is missing in NAS-type plants. The observation that glucosibarin levels stay constant and that NAS-type plants still contain minute levels of glucobarbarin, show that there is still some hydroxylation activity in NAS-type plants. Possibly, 2-*R* hydroxylation, leading to glucosibarin, is performed by another enzyme, or is performed in another, unaffected, region of the same enzyme that performs the 2-*S* hydroxylation, resulting in glucobarbarin.

Difference with other polymorphisms in B. vulgaris

The polymorphism reported here differs from the G-type/P-type polymorphism discovered in Denmark. Agerbirk et al. (2003b; 2003a) reported two types of *B. vulgaris*, that differed in a set of chemical, biological and morphological characters, including glucosinolate polymorphisms. The Danish polymorphism included differences in flea beetle- and diamondback moth resistance, leaf pubescence (Agerbirk et al., 2003a), and saponin content (Agerbirk et al., 2003b). Both types were identified as *B. vulgaris* var. *arcuata* based on traditional morphological criteria. However, the G-type chemically did not differ from *B. vulgaris* var. *vulgaris*. The provisionally named 'P-type" (for **p**ubescent) apparently has not been previously recognised by modern botanists. Neither of the above mentioned Danish types had a high content of gluconasturtiin in the leaves, nor did one of the Dutch *B. vulgaris* in our experiments produce trichomes. We did not study whether the plants from the

Netherlands would morphologically belong to var. *arcuata* or var. *vulgaris*, as this varietal difference is not generally recognised as significant by Dutch and Anglosaxon botanists. The relatively significant amounts of glucosibarin in NAS-type plants may indicate a partial similarity to P-type plants, but we expect it to be a coincidental similarity. The distinct differences in glucosinolate profiles between the two Dutch chemotypes is yet another proof of the high polymorphy present within the species *B. vulgaris* (Lawalrée, 1955).

Conclusion

In this paper we have shown that the differences between the common BAR-types and the rare NAS-types are consistently expressed in the plants, are present in approximately half of the Dutch populations, and are inherited via a simple Mendelian inheritance. Moreover, gluconasturtiin and glucobarbarin and their breakdown products have the potential to affect different species of aboveground and belowground feeding herbivores. Additionally, *B. vulgaris* is closely related to the model crucifer *A. thaliana* which allows the application of molecular tools designed for *A. thaliana* for molecular genetic analysis of *B. vulgaris.* These aspects make this specific glucosinolate polymorphism an outstanding candidate for studying both the molecular genetic as well as the ecological-evolutionary aspects of chemical diversity in plants (Ober, 2005). Eventually, it will depend on the relative frequency of herbivores in natural *B. vulgaris* populations as well as on the impact of these herbivores on plant fitness, whether one of the chemotypes will experience a selective advantage, or whether the polymorphism is selectively neutral. Because both root and shoot glucosinolate compositions differ between the two chemotypes, it is important to consider the effects of root as well as shoot herbivores and their natural enemies (Van der Putten et al., 2001).

Experimental

Plant rearing

For our experiments we used the crucifer *Barbarea vulgaris. B. vulgaris* has a wide native distribution area (Eurasia) and is furthermore introduced to North America, Africa, and Australia where it appears as a noxious weed. It grows mainly in grassy places along roads, rivers and ditches. *B. vulgaris* is a biennial or perennial that forms a rosette in the first year, while during the second year it produces one or more flowering stalks (Hegi, 1962). The plants were grown in a glasshouse, temp. was 21 °C (day) and 16 °C (night), with a relative humidity of 60%. Natural daylight was supplemented with sodium lamps to maintain the minimum PAR at 225 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a photoperiod of 16:8 (L:D). Seeds were surface sterilised in 1% NaClO and germinated on glass beads. Ca. one week after germination, seedlings were transplanted to a mixture of peat soil (Potgrond 4, Lentse Potgrond BV., Lent, The Netherlands) and 20% sand. After 2 weeks the seedlings were transplanted to 1 l pots. They were watered daily and fertilized regularly with half strength Hoagland's nutrient solution with a doubled KH_2PO_4 content.

Consistency of the chemotype in different organs and induced plants

To check consistency of the chemotype throughout the plant we analysed the root, rosette leaf, stem leaf and flower of the same flowering plant (n = 3 per chemotype). Seeds with the same type as the mother, obtained by crossings of these plants, were analysed individually (n = 6 per BAR-type plant; n = 5-6 for NAS-type plant, except for one NAS-type plant which did not produce any seeds). All samples, except seeds, were frozen at -20 °C, lyophilized and stored at -20 °C. Glucosinolate data were arcsine-square root transformed to meet assumptions of normality and homogeneity of variances and analysed by Analysis of Variance (ANOVA), with organ and chemotype as fixed factors. Differences between types per organ were tested with a Tukey post-hoc test.

Since leaf samples taken in natural populations are from plants that may be damaged above- or belowground by herbivores, we checked whether root or shoot induced responses affect the classification of the chemotypes. Seeds from 2 *B. vulgaris* lines that were known to yield either only NAS or only BAR-types were germinated and grown on 1.3 l pots filled with sand. The plants received 2P-Hoagland solution as described in van Dam et al. (2004). After 8 weeks, the rosettes were randomly distributed over 3 treatment groups (n = 8-10 per group). One group received 500 µg jasmonic acid (JA) to the shoots (SJA), one the same amount of JA to the roots (RJA) and a third group was treated with acid water (pH set to 3.7 with 1 N HCl) to control for effects of acid application (for details, see Van Dam et al., 2004). Seven days later, the shoots of the plants were harvested, frozen, lyophilized, ground to a fine powder, and extracted as described below. The resulting glucosinolate concentrations were arcsine-square root transformed before analysis by Multivariate ANOVA with JA treatment and type as main factors, followed by protected contrast analysis and univariate ANOVAs to examine differences between individual treatment groups in more detail.

Geographical distribution

The chemotype distribution of *B. vulgaris* was analysed from 2002 till 2005. Plants and seeds were collected from 27 natural populations in The Netherlands and surrounding countries (Table 2.1). The populations were surveyed by collecting a stem leaf from up to 50 flowering plants per population. Because a single *B. vulgaris* plant can have multiple flowering stems, leaf samples were taken at least 1 meter apart from each other to avoid sampling from the same individual. Additionally, we tested a commercial batch (provided by “de Morgenster”, Van Galenlaan 20, 1403 TS, Bussum, The Netherlands) which was collected in a population near Leunen (51.52 °N; 5.98 °E), and a second commercial batch (provided by “De Bolderik”, Floralaan 108, 1693 GR, Wervershoof, The Netherlands) which had an unknown geographic origin. From each commercial batch we analysed 25 seeds or seedlings. For the analysis of glasshouse grown seedlings, we collected one or two of the first fully developed leaves that contained lobes.

Chemical analysis

Glucosinolates were extracted from ground samples with 70% MEOH, desulphated with arylsulphatase (Sigma, St. Louis, IL, USA) on a DEAE-Sephadex A 25 column (EC, 1990) and separated on a reversed phase C-18 column on HPLC with a CH_3CN-H_2O gradient as described in Graser et al. (2000). Glucosinolate analysis was performed with a PDA detector (200 – 350 nm) with 229 nm as the integration wavelength. Sinigrin (sinigrin monohydrate, ACROS, New Jersey, USA) was used as an external standard. We used the correction factors for detection at 229 nm from Buchner (1987) to calculate the concentrations of the glucosinolates (Van Dam et al., 2004).

Desulfoglucosinolate peaks were identified by comparison of HPLC retention times and UV spectra with authentic standards isolated from *B. vulgaris* as previously derscribed (Agerbirk et al., 2001b), as well as standards kindly provided by M. Reichelt, MPI Chemical Ecology, and a certified rape seed standard (Community Bureau of Reference, Brussels, code BCR-367R). For glucobarbarin, we used the same response factor as for gluconasturtiin. For screening the type distribution in natural populations and in the crossing experiments, no exact quantities of the glucosinolates were needed. Therefore we shortened the method for chemotype determination by omitting the freeze-drying of the eluate after desulfation. When the peak area of glucobarbarin divided by the peak area of gluconasturtiin was > 10, the plant was considered a BAR-type, when this ratio was < 0.1 it was considered a NAS-type. Less than 1% of the samples could not be clearly classified as either type, which we could ascribe mainly to errors in the extraction procedure. These samples were excluded from further analyses.

Inheritance of the chemotype

To check if the chemotype was heritable we selected 9 pairs of plants (9 NAS plants and their "nearest neighbour" BAR plants) from the natural population "Elderveld". In May 2002 the flowering plants were transplanted to an experimental garden. Seeds of these parent plants were collected and sterilised in 1% NaClO, stratified at 5 °C, and germinated. Of the five pairs of neighbouring BAR and NAS plants that yielded sufficient viable seedlings (n = 58 ± 9.63 SE) the chemotype of the half-sib offspring (F1) was analysed individually by taking a sub-sample (two leaves) for each plant. The G-test of independence was used to evaluate whether the chemotype of the offspring was independent of the maternal chemotype.

To test simple Mendelian inheritance we performed a series of crosses. Plants used for crosses were reared from seeds of *B. vulgaris* from natural populations (Table 2.1) in 2002-2003. We selected the following plants: (A) From Elderveld we used the above described half-sibs of which the maternal plant was NAS-type and had offspring of both types. Crosses were performed with 5 NAS and 6 BAR individuals from three half-sib families. (B) From Heteren, seeds of several individuals were bulk collected. After germination and type determination we used the plants derived from these seeds for the crosses. Only 2 crosses succeeded. Both were selfings of BAR-type plants. (C) From the Oosterbeek population we screened and labelled 50 plants. Type determination revealed only 1 NAS plant. Seeds from this plant were collected, germinated and analysed. This yielded 80% BAR-type plants. 2 BAR and 2 NAS plants of this half-sib family were selected for further crossing. Plants were grown in 1 l pots and vernalized at 5 °C and 10 hours light for minimally 12 weeks, and then

transplanted into 2 l pots to enhance growth and flowering. The crossings were performed by rubbing ripe pollen of the pollen donor with an eye make-up applicator directly on the ripe stamen of a labeled, emasculated flower on the receiving plant. All crosses were performed reciprocally and receiving flowers were emasculated prior to anthesis to avoid selfing (for details see Van Dam et al., 1999).

Even though *B. vulgaris* has been reported to be a self-compatible plant (Lawalrée, 1955), selfings yielded very few viable seeds. Therefore, we used half-sib plants with similar types to perform crosses between similar chemotypes. First we crossed NAS-type half-sibs (F1) from a NAS-type mother (P), with each other (Table 2.2, cross 1-10). Subsequently we crossed two heterozygous half-sibs (Table 2.2, cross 11-18). After this first round of crosses the parental plants were transplanted to the experimental garden and used later for back-crossing heterozygous plants into a NAS plant to check a 1:1 ratio. Of the F2, resulting from these backcrosses 16-25 seedlings were analysed (Table 2.2, cross 19-26). Finally we selfed BAR-type plants from the "Heteren" population (96% BAR) (Table 2.2, cross 27-28). BAR-type offspring was transplanted into the experimental garden where they could randomly cross with BAR- and NAS-type plants (Table 2.2, cross 29-30).

For statistical analyses of the crossing experiments we used the replicated goodness-of-fit test (G-statistic). $G_{heterogeneity}$ was used to check whether the ratios of the progenies (replicates) were homogeneous. G_{pooled} was calculated for all replicates together. The two G values sum up to a G_{total} that was used to test whether the data fit the expected ratios of glucosinolate types in the offspring (Sokal and Rohlf, 1997). The results for the reciprocal crosses were pooled because they did not significantly differ (Chi square test: $P > 0.4$).

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CHAPTER 3

Metabolic and developmental costs associated with a *Barbarea vulgaris* glucosinolate polymorphism

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Abstract

Heritable defence polymorphisms have been found in several plant species. Evolutionary theory predicts that these polymorphisms may be maintained if the costs and benefits of producing different defence compounds are balanced. Here we analyse the costs associated with a heritable glucosinolate polymorphism in *Barbarea vulgaris*. The dominant phenotype produces predominantly glucobarbarin (BAR-type), whereas the recessive type mainly produces gluconasturtiin (NAS-type). Using seeds of two half-sib families with mixed progeny we investigate whether the chemotypes differ in direct costs on the level of plant metabolome, growth and development. Secondary metabolites were analysed comprehensively using untargeted LC- QTOF-MS analysis. The resulting metabolomic profiles confirmed that the chemotypes mainly differ in their glucosinolate composition and far less with respect to other metabolites. Moreover, these differences were stronger in shoots than in roots. The levels of saponins and flavonoids, which are known to affect herbivory as well, differed between the half-sib families, however, not between the chemotypes. Experiments under controlled conditions on several half-sib families and in the absence of herbivores showed that growth rates and development were often affected by the genetic origin of the seeds. The only difference between the chemotypes was that seeds from BAR-type plants weighed less than seeds from NAS-type. However, this did not translate into a lower germination rate or germination success, smaller seedling size, lower rates of biomass accumulation or a different phenology. Both chemotypes produced similar numbers of flower stalks. Thus, despite the clear differences in glucosinolates, the chemotypes did not differ in growth or development, nor did they show large differences in metabolite profiles. Therefore, we conclude that there are no direct metabolic costs to these two chemotypes when compared to each other. Hence, the existence and maintenance of the glucosinolate polymorphism in natural *B. vulgaris* populations may be explained by specific local herbivore frequencies/loads, rather than by costs related to plant physiological processes.

Keywords

herbivore defence, germination, LC-QTOF-MS, metabolomics, Principal component analysis (PCA); Partial Least Squares Discriminant Analysis (PLS-DA)

Introduction

Discrete and heritable polymorphisms have been demonstrated for a number of plant defence traits in different natural plant species (Linhart and Thompson, 1999; Van Dam et al., 1999; Van Dam and Baldwin, 2003; Van Leur et al., 2006). The relative frequencies of the different genotypes in natural populations are supposed to be determined by the balance of costs and benefits associated with each phenotype. In case of defence compounds the benefits, *i.e.* increased resistance against one or more herbivores or pathogens (Simms and Rausher, 1987; Strauss et al., 2002), are generally much better studied than their costs. However, a proper understanding of how chemical polymorphisms arise and are maintained in natural populations requires investigation of the costs of these defences.

We identified a heritable glucosinolate polymorphism in *Barbarea vulgaris* (Leur et al. (2006). Glucosinolates and their breakdown products affect plant antagonists directly by being noxious or unpalatable and indirectly by attracting natural enemies of herbivores (Mithen, 2001a; Wittstock et al., 2003). The *B. vulgaris* glucosinolate polymorphism consists of two heritable chemotypes. The most common chemotype has a glucosinolate profile which is dominated by glucobarbarin (2-*S*-OH-2 phenylethylglucosinolate; BAR-type plants), whereas in the less abundant chemotype gluconasturtiin is most prominent (2-phenylethylglucosinolate; NAS-type plants). We assume that in BAR-type plants the gluconasturtiin is hydroxylized by an enzyme belonging to the 2-oxidoacid dioxygenase enzyme family, similar to the enzyme responsible for the conversion of butenyl glucosinolate into 2-OH-butenyl glucosinolate (progoitrin) (Hall et al., 2001; Kliebenstein et al., 2001b)

Due to the differences in glucosinolate structure and their concomitant breakdown products, the chemotypes have different effects on their biotic environment. This was shown for the aboveground generalist herbivore *Mamestra brassicae* and for the belowground specialist *Delia radicum* (Chapter 4 and 5 this thesis.). Whereas *M. brassicae* performed better on NAS types, *D. radicum* performed better on the BAR type (Chapter 4 and 5, this thesis). Additionally, we found that several other herbivores differentially preferred BAR-type or NAS-type plants in an experimental garden experiment (Chapter 6, this thesis). Eventually, these differential preferences within the herbivore community may result in equal herbivore loads for the two types. Therefore, it is important to assess whether costs associated with the production of the glucosinolates may contribute to the maintenance of this polymorphism.

Costs associated with production of defence compounds can be direct or indirect. Direct costs are costs associated with plant physiological processes which reduce growth, survival or reproduction. Indirect costs, also named ecological costs, arise if resistance against one organism changes interactions with other species in the natural environment, resulting in a fitness decrease. This can happen if increased defence levels decrease attractiveness for mutualists such as pollinators and symbionts, reduce intra- or interspecific competitive ability, increase attractiveness to specialist herbivores, or reduce tolerance to biomass loss (Strauss *et al.*, 2002). In the present study, we aim to assess direct costs of the above *B. vulgaris* glucosinolate polymorphism.

Direct costs are often referred to as allocation costs, under the assumption that the limited availability of energy and substrates causes trade-offs between the production of the defence compound and other physiological processes (Bergelson and Purrington, 1996). Substrate limitation can occur when precursors or intermediates are used in several processes. Phenylalanine, for example, is the rate-limiting precursor for phenylpropanoid biosynthesis (Hartley et al., 2000; Celenza, 2001) and tryptophan for biosynthesis of indole glucosinolates (Grubb and Abel, 2006). Both precursors are at the same time essential amino acids for protein synthesis. Consequently, alkaloid and glucosinolate syntheses compete directly with protein synthesis and growth.

Direct costs of glucosinolate production may also be caused by pleiotropic effects on other aspects of plant performance caused by genes conferring resistance (Simms, 1992). First, pleiotropic costs can be due to detrimental linkage. The chance of having associated traits in linkage disequilibrium (e.g., non-random associations between alleles at physically unlinked loci), increases when plants have limited variation in genetic background. This is especially relevant when using recombinant inbred lines of extensively selfed plants such as *Arabidopsis thaliana* (Parker, 1992). However, even obligatory out-crossing species may not always mate at random and thus might also suffer from pleiotropic effects caused by linkage disequilibrium. If strong linkages between resistance and other traits are favoured by natural selection, they represent legitimate sources of (at least current) costs of resistance in natural populations (Strauss *et al.*, 2002). Next to detrimental linkage, other pleiotropic effects can be costly as well (Mitchell-Olds and Bradley, 1996). Up- or down-regulation of a certain pathway to increase resistance can, for example by changing metabolic feedback loops, disrupt related metabolic pathways (Lenski, 1998). For instance, the synthesis of two tryptophan derived metabolites, indole glucosinolates and indole-3-acetic acid (IAA), are closely connected (controlled by a common Myb transcription factor). A decreased synthesis of the indole glucosinolates in an *A. thaliana* knock-out mutant resulted in an increased IAA production, thus altering the plant growth rates/phenotype (Celenza *et al.*, 2005).

We envision several ways in which pleiotropy can cause differences in metabolite profiles between the two *B. vulgaris* chemotypes. Assuming that the putative 2-ODD enzyme is absent or inactive in NAS-type plants, the possible side products of that enzyme may also be lacking in those plants. We also observed that BAR plants overall have higher glucosinolate levels in their leaves than NAS plants (Van Leur et al., 2006). This may indicate that gluconasturtiin has a negative feedback on earlier steps in the biosynthesis. Disruption of the pathway towards glucobarbarin production thus may result in altered levels of precursors or products of related pathways.

Our first hypothesis is that the *B. vulgaris* chemotypes have differential metabolite profiles due to metabolic pathway disruption or other pleiotropic effects of the changes in gene function. Whether such metabolic differences are present in this glucosinolate polymorphism, may be elucidated by studying the metabolic profiles of BAR-type and NAS-type plants. To identify metabolites that consistently (*i.e.* over two half-sib families) differ between BAR-type and NAS-type plants, we combined an untargeted method for metabolite analyses (LC-QTOF-MS) with supervised and unsupervised statistics. Additionally, we analyzed whether the chemotypes differ in levels of some compounds known to be present in *B. vulgaris* and of compounds that are involved in plant defence as well as some common plant metabolites.

Our second hypothesis is that differences between the chemotypes in production of glucosinolates -and possibly of other metabolites- may result in differential growth and development of the plants. For example, the higher amount of glucosinolates in the BAR-type seeds and rosette leaves may lead to higher allocation costs and consequently lower growth rates of BAR-type than of NAS-type plants. As the chemotype of *B. vulgaris* is already determined in the seed (Van Leur et al., 2006), we measured growth from seeds to flowering plants under controlled conditions. By comprehensive analysis of metabolites on one hand, and growth and development of the two chemotypes on the other hand, we analyzed two important aspects of direct costs associated with the glucosinolate phenotypes in *B. vulgaris*. We discuss how direct costs of this glucosinolate polymorphism may contribute to explaining the relative abundance of both chemotypes in natural populations.

Materials and Methods

Plant material

B. vulgaris is native in Eurasia and is introduced to North America, Africa, and Australia where it is a noxious weed. It grows mainly in grassy vegetation along roads, rivers and ditches. *B. vulgaris* is a biennial or short perennial that forms a rosette in the first year, and produces one or more flowering stalks in the second year (Hegi, 1962). We collected seeds from two natural populations of *B. vulgaris* in the Netherlands: Elderveld (EL, 51.95 °N; 5.87 °E) and Heteren (HE, 51.95 °N; 5.77 °E). The EL population consists of 22% NAS-type plants and the HE of 4% NAS-type plants (Van Leur et al., 2006). The seeds were collected from individual BAR-type and NAS-type maternal plants, which had been freely (cross)-pollinating in their natural populations.

Glucosinolate type determination

To determine the chemotype of each individual, either the youngest fully grown leaf (growth experiment) or the seed or seedling at the end of the germination experiment, was sampled and frozen at -20 °C, lyophilized and stored at -20 °C until analysis. Glucosinolates were extracted from ground samples with 70% MeOH, desulphated with arylsulphatase (Sigma, St. Louis, IL, USA) on a DEAE-Sephadex A 25 column (EC, 1990) and separated on a reversed phase C-18 column on HPLC with a CH_3CN–H_2O gradient as described in van Dam and Raaijmakers (2006). Glucosinolate analysis was performed with a PDA detector (200–350 nm) with 229 nm as the integration wavelength. Desulfoglucosinolate mass signals were identified by comparison of HPLC retention times and UV spectra with authentic standards isolated from *B. vulgaris* as previously described (Agerbirk et al., 2001b), as well as standards kindly provided by M. Reichelt, MPI Chemical Ecology, and a certified rape seed standard (Community Bureau of Reference, Brussels, code BCR-367R). For glucobarbarin, we used the same response factor as for gluconasturtiin. When the peak area of glucobarbarin divided by the peak area of gluconasturtiin was > 10, the plant was considered a BAR-type, when this ratio was < 0.1 it was considered a NAS-type. The < 1% of the samples that could not be classified clearly as either type was omitted from the experiments and statistical analysis.

Metabolomics analysis

For metabolomic analysis, we use reversed-phase Liquid Chromatography coupled to Quadrupole Time-Of-Flight Mass Spectrometry (LC-QTOF-MS) (De Vos *et al.*, 2007). Generally, LC-MS is more sensitive than Nuclear Magnetic Resonance (NMR) and enables the detection of a large group of plant secondary metabolites such as alkaloids, saponins, phenolic acids, phenylpropanoids, flavonoids, glucosinolates, polyamines and derivates thereof (Huhman and Sumner, 2002; Moco et al., 2006; Rischer et al., 2006).

Plants from two half-sib families with homozygous NAS-type maternal plants EL8 (offspring 48% BAR) and EL22 (offspring 65% BAR) were grown in a glasshouse (21 °C/16h L; 16 °C/8h D). During the day, natural daylight was supplemented with sodium lamps to maintain the minimum PAR at 225 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Plants were watered daily to maintain soil humidity at 14% (w/w) and regularly fertilized with half strength Hoagland's nutrient solution with a doubled KH_2PO_4 content.

After 3 weeks, one leaf of every plant was sampled for type determination. Ten BAR-type and ten NAS-type plants per family were selected for untargeted metabolite analysis. Roots and shoots of six-weeks-old plants were harvested, lyophilized and ground. Of each of the 80 samples, 50 µg (± 1) was transferred to 15 ml glass tubes and extracted with methanol and analyzed on LC-PDA-QTOF-MS, essentially as described in De Vos et al (2007). A Synergi Max-RP column (2.0 × 250 mm, 4 µm pore size, 40 °C) and a gradient of 5 to 75% acetonitril (acidified with 0.1% formic acid) in 45 min. was used. Mass signals were subsequently extracted and aligned using Metalign software (www.metalign.nl). Mass signals were removed from the dataset when they were absent in half of the samples of each chemotype/family combination. Mass signals indicating saturation of the MS detector were also removed. A total of 71 mass signals were saturated and these mass signals could all be assigned to the parent and some isotope ions of some specific abundant metabolites: gluconasturtiin, glucobarbarin, glucosibarin, glucobrassicin, neoglucobrassicin, or flavonoid 3,4 and 7 (see Table 3.3). Instead of these saturated ions, corresponding non-saturated isotopic signals were used for statistical analyses.

Chemometric modelling and statistical analysis of metabolomics data

Because the metabolomic analyses provide information on a large number of metabolites, including their different isotopes, considerable correlations between the mass signals are expected. Therefore, multivariate chemometric analyses designed to deal with these correlations are required to extract the metabolic information from the data. Initially, the metabolic variation between the different plants was visualised using Principal Component Analysis (PCA). Hereby, the dimensionality of the dataset was reduced while retaining the characteristics of the dataset that contribute most to its variance (Jolliffe, 2002). The differences between both chemotypes were visualised in PCAs per family (percentages of explained variation see Table 3.1), and could be established by extending the PCA with a supervised regression to maximize the separation between the chemotypes by fitting Partial Least Squares for Discriminant Analyses (PLS-DA) models (Barker and Rayens, 2003). Each PLS-DA model was validated with permutation test to detect the percentage of random models performing equally well (Rubingh *et al.*, 2006). By plotting the PLS-DA discriminant function (*i.e.* the regression coefficients) of family EL8 against that of EL22, the conserved

chemotype effects between families can be identified. Multivariate analyses were performed using Matlab version R 2006 B (Mathworks™ , Inc. Natick, Massachusetts, USA).

Significant differences in the expression of selected compounds that are important for chemotype differentiation (encircled dots in Figure 3.1a and 1b) and known *B. vulgaris* compounds were tested in separate Mann-Whitney-U tests for type and family effects, using non-transformed data. As flavonoid 1 showed an unexpected chemotype effect, this chemotype effect was additionally tested separately for family EL8 and EL22 using Mann-Whitney-U tests.

Seed biomass

To determine seed biomass from all allele combinations, we selected seeds from half-sib families and from selfings. We selected two half-sib families with homozygous BAR-type maternal plants HE49 and HE35 (offspring 100% BAR), two half-sib families with homozygous NAS-type maternal plants EL8 (offspring 48% BAR) and EL22 (offspring 65% BAR), and two half sib families with heterozygous maternal plants EL9 (offspring 73% BAR) and EL14 (offspring 79% BAR) from BAR type maternal plants. To obtain 100% NAS-type offspring, we self-fertilized two NAS-type maternal plants (14 and 15). All seed batches will be named "families" from here onwards. Of the families with 100% BAR-type or 100% NAS-type offspring, we directly weighed 5 groups of 10 seeds. Of the mixed families we weighed and labelled 96 seeds and determined the chemotype at the end of the germination experiment.

Germination

Germination rate and germination success was analyzed by monitoring individual seeds of the above described families EL8, EL22, EL9 and EL 14. Of each family 96 seeds were placed individually in wells of sterile 24 wells cell culture plates (TPP© AG, Zollstrasse 155, 8219 Trasadingen , Switzerland) filled for 2/3 with sterile glass beads and demineralised water. During the experiment, the plates were kept in a controlled climate chamber (21 °C/16h L; 16 °C/8h D). During 13 days, we checked the developmental stage of the seed, using a binocular. Water was added when necessary. We distinguished and noted the following visible germination stages: (1) seed coat bursted, (2) root tip visible, (3) cotyledon visible (4) primordium of first pair of true leaves visible. At the end of the experiment we determined the chemotype of the seeds or seedlings.

Growth and development

To compare growth rates between the chemotypes, we selected, based on the BAR/NAS ratio in the offspring, two of the above described families (EL8 and EL22). To avoid sampling to affect growth rates, leaves were sampled for chemotype determination only when plants were harvested for biomass determination or at the end of the experiment. Seeds were sown on seedling trays filled with sand (day 0). After 16 days, the seedlings were transplanted into 1.5 litre pots containing sand. The pots were randomly distributed in the greenhouse to randomize effects of heterogeneous abiotic factors. The plants were grown as

described above for the Metabolomics experiment. We performed measurements at different developmental stages, starting with the seed mass prior to sowing at day 0. Seedling specific parameters (maximum width and length of cotyledons of all plants) were measured non-destructively at day 16. Of each rosette plant (day 16-163) we measured the number of leaves, the length of the longest leaf at days 16, 30, 37, 44, 51, 58 and 65. Root length, fresh- and dry mass of roots and shoots were measured at destructively harvested subsets of 10 plants per family at days 16, 30, 37, 44, 65. Of the plants harvested at day 44, we additionally measured the thickness of the root-shoot junction and measured the total leaf area using Delta-T-Scan® software version 2.03 (Delta T Devices, Cambridge, UK, Kirchof,G., and Pendar, K. 1993)

From day 65 till day 163 plants were placed at 4 °C to induce flowering. The reproductive stage of the plants is represented in the measurements from day 163 till the end of the experiment (day 203). At day 163, 165, 167, 168, 170, 172, 173, 175, 178, 180, 185, 190, 196 and day 203 we counted number of flower stalks, measured the length of the flower stalks, recorded when there were small flower buds in the rosette, and when the flowers opened. At day 178 we additionally harvested 10 plants per batch for destructive measurements of root length and biomass of roots, shoots and flower stalks.

Statistical analyses growth and germination

Statistical analyses were performed using STATISTICA (data analysis software system), version 8.0 (StatSoft, Inc., Tusla, OK, USA). Normality and homogeneity of variance were checked by inspection of the residuals. When data did not fulfil the assumptions, they were either log-transformed or a non-parametric test was applied.

Seed weights of heterogeneous batches were analysed using factorial ANOVA with factors type and family. For homogeneous batches the family effect was tested in a one-way ANOVA and the type effect using a Mann-Whitney U test. Type and family effects, and their interaction, on seedling development were tested using separate factorial ANOVAs for visibility of seed coat, root, cotyledon and primordium of the fist pair of true leaves. Seedling characteristics, total leaf area at day 44 and number and total length of the flower stalks were measured only at one day and tested using factorial ANOVAs with type and family as factors. Dry mass of roots, shoots, total dry mass and the shoot/root ratio were tested several times but on different plants and were therefore analysed using MANOVA protected univariate ANOVAs including time, type and family. As the other leaf characteristics (number of leaves, maximum leaf length and number of leaf lobes) were measured non-destructively at several time points, they were analysed using repeated measurement ANOVAs.

Results

Metabolic variation between chemotypes and families

PCA on LC-QTOF-MS mass signals showed that for shoot as well as for root metabolite profiles, a considerable percentage of the variation could be explained by chemotype and also by family (Table 3.1). This contribution of family as well as chemotype was confirmed in subsequent univariate ANOVAs on shoot data (number of significant mass signals for type = 4539, for family = 2695, and for type × family interaction = 1534, overlap family and type = 729, number of significant mass signals expected by chance: 5% × 17728 = 887 mass signals) but for root data only family showed more significant mass signals than could be expected by chance (type = 395 mass signals, family = 1860 mass signals, type × family interaction = 367 mass signals, overlap family and type = 143 mass signals: chance: 5% × 7049 = 352 mass signals). The substantial variation between the families required a separate analysis of type effects for each family.

Table 3.1 Percentage of variation explained by the two principle component axes, which best separated family and chemotype in an overall PCA analyses and in separate analyses on chemotype within family EL8 and within family EL22 on root and shoot LC-QTOF-MS mass signals.

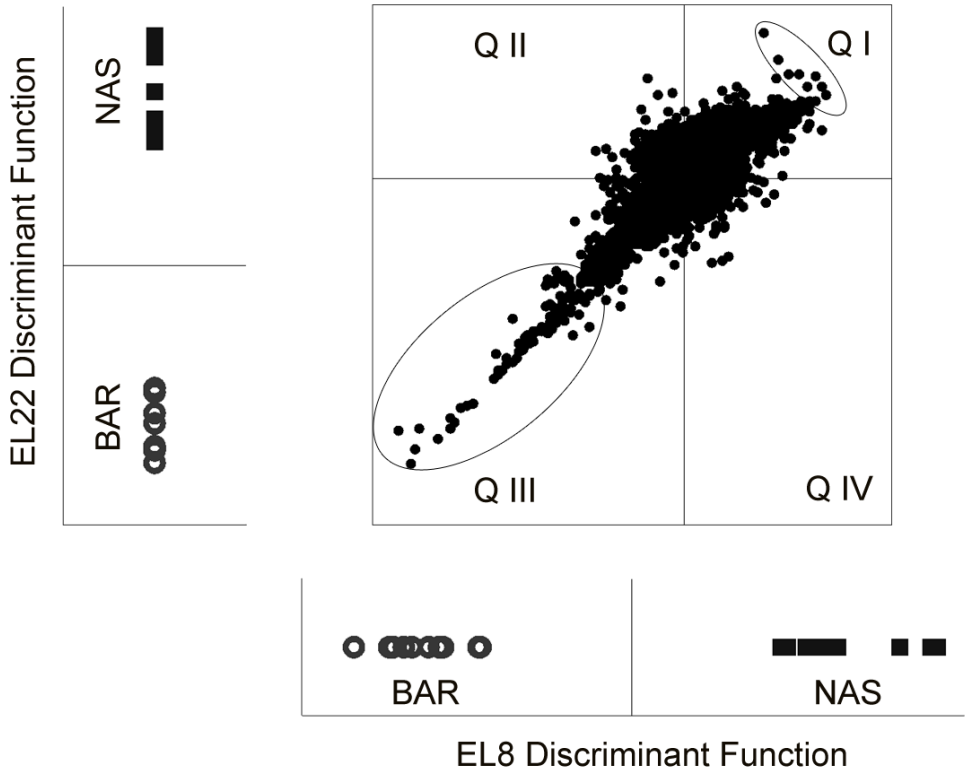
	family		**chemotype**					
	overall		overall		EL8		EL, 22	
	Major PC	Minor PC	Major PC	Minor PC	PC1	PC2	PC1	PC2
shoot	PC2 = 11	PC3 = 9	PC1 = 17	PC2 = 11	24	13	20	15
root	PC1 = 34	PC4 = 5	PC2 = 9	PC3 = 6	28	14	34	9

Comparison of chemotype metabolite profiles

PLS-DA models run on root and shoot data per family clearly discriminated both chemotypes with 1 or 2 latent variables. Permutation tests showed that the discrimination within EL8 was good (only 5% of random models also predict class membership perfectly in shoots and 4% in roots) and within EL22 was moderate to good (15% in shoot and 13% in roots) .

Our main interest is in variation between the chemotypes that is consistent between the two families. This chemotype specific variation was observed by plotting both discriminant functions against each other. For shoots (Figure 3.1a) and for roots (Figure 3.1b) all mass signals either were close to centre of the graph, or were positioned on a line from down-left (quadrant III = Q III) to the upper-right (Q I). The mass signals in the cloud around the centre represent metabolites that did not have a high discriminant function value in either of the families, and thus had no high contribution to the discrimination of BAR-type and NAS-type samples. The absence of points in the far ends of the figure axis or the far ends of Q II and Q IV reveals that there were no metabolites that contributed highly to the chemotype discrimination in one family, but not in the other. Hence, the metabolites that highly contributed to the BAR-NAS separation were similar for both families. The larger number of signals with a high loading in Q III indicates that in BAR-type samples more compounds with higher concentrations contributed to separation of the chemotypes.

A



B

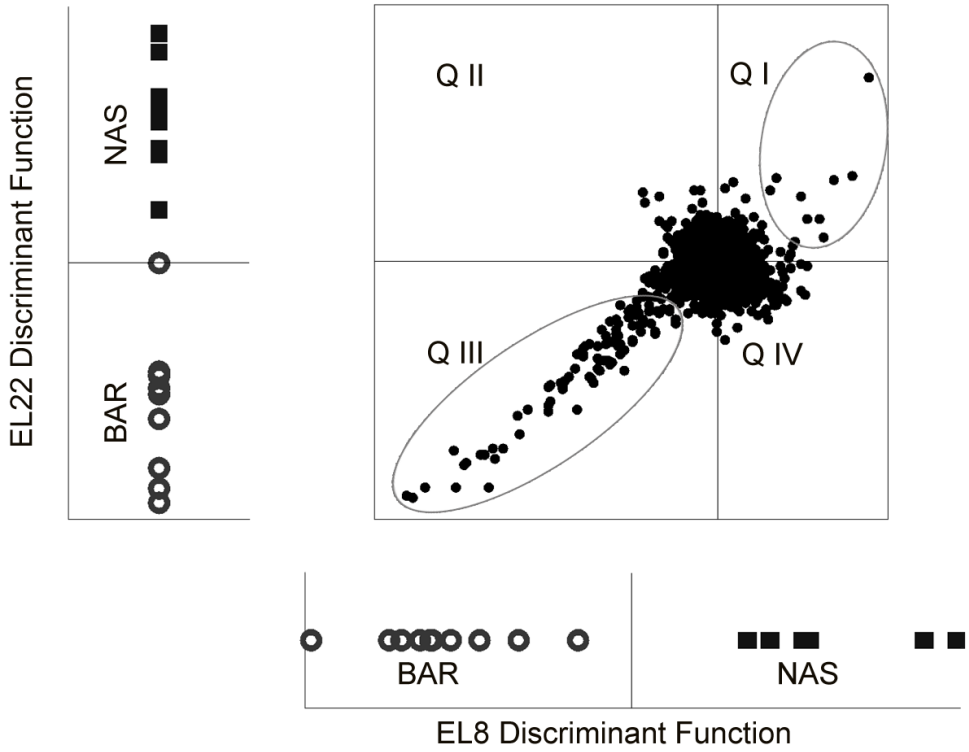


Figure 3.1 Discriminant functions (PLS-DA) of *Barbarea vulgaris* LC-QTOF-MS metabolite shoot (A) and root (B) data of half-sib family EL8 (x-axis) and EL22 (y-axis). On the axes, the BAR-type samples are depicted as open circles and NAS-type samples by squares. The encircled dots were selected for univariate analyses.

Assignment of the differential mass signals

To identify the metabolites that consistently differ between BAR-type and NAS-type plants we selected those mass signals that could be clearly discriminated from the cloud of mass signals around the centre (see encircled dots in Figure 3.1). This resulted in 4 groups of mass signals: shoot Q III (92 mass signals), shoot Q I (10 mass signals), root Q III (50 mass signals) and root QI (11 mass signals). First, we analysed the correlations between these mass signals by cluster analysis. Mass signals closely clustering together and with similar scan numbers (retention times), were assigned to one metabolite. Using these clusters, exact mass data, scan numbers and chromatographic absorbance spectra of the mass signals were assigned to (putative) metabolites.

Although many glucosinolate mass signals were removed from the data set because they produced saturated signals, 86 mass signals (92% of the selected mass signals in root and shoot Q III) represented glucobarbarin/glucosibarin (BAR/SIB). As these two glucosinolates have the same exact masses and coeluted in this analysis, they could not be distinguished. Moreover, the BAR/SIB chromatographic peaks were generally highly saturated, which caused peak broadening and thereby misalignment of masses belonging to the same compound. To compare BAR/SIB content between the chemotypes, we therefore summed the mass signals of mass 438 from scans 861-915 in shoots and scans 875-894 in roots. BAR/SIB was saturated in all BAR-type samples, and absent (or within the noise range) in all NAS-type samples. Similarly, in the selected signals from shoot Q I, 80% (8 mass signals) of the mass signals represented gluconasturtiin.

The remaining mass signals, which did not represent BAR/SIB or gluconasturtiin, were analysed univariately (Table 3.2). All shoot mass signals selected from Q I and Q III indeed differed highly significantly between the chemotypes but not between the families. Many of the mass signals were only present in one of the two chemotypes. As could be expected from the lower fit of the root data model, root mass signals showed a less significant difference. Three metabolites were not significantly different and only one metabolite was unique for NAS-type plants. Still the selected mass signals showed no significant difference between the families. The significant mass signals comprised three BAR/SIB related metabolites and 8 mass signals of which we could not yet identify the metabolite (Table 3.2).

Glucosinolates were represented by a relatively high number of discriminating mass signals and may consequently have had a relative high influence on the data analyses. Therefore, we re-ran the analyses omitting all scans in the BAR/SIB region (856-915) and in the NAS region (1166-1195). This reduction of the dataset also caused elimination of some non-glucosinolate compounds from these scan regions. Both chemotypes could not be distinguished based on this reduced metabolic information.

Table 3.2 Relative abundance and Mann-Whitney U statistics (Z-values and significance levels: *$P < 0.05$, ** $P < 0.001$) on mass signals of LC-QTOF-MS derived metabolites from shoot and root tissue of two *Barbarea vulgaris* chemotypes (Type) of two families. Metabolites were selected based on high loadings in a PLS-DA analyses, provided that the direction was consistent between the two families.

					EL8		EL22	
Group	**Description**	**Measured Mass**	**Type**	**Family**	**BAR**	**NAS**	**BAR**	**NAS**
Shoot								
BAR	BAR-SIB related 1	227	-5.20**	-0.12	121	0	122	0
	unknown 1	327	-5.20**	-0.27	188	0	268	0
	unknown 2	1442	-5.20**	-1.42	241	11.5	152	0
	unknown 3	1285	5.07**	0.82	105	0	91	0
	unknown 4	600	-5.20**	-0.61	28	473	110	448
	unknown 5	556	-5.20**	-0.52	184	0	157	0
NAS	BAR-SIB related 2	1078	5.01**	-1.3	38	160	0	125
	BAR-SIB related 3	1313	-5.20**	-0.61	970	66	636	89
Root								
BAR	unknown 6	383	2.28*	-1.07	60	32	83	40
	unknown 7	768	2.95*	0.88	73	30	58	25
	unknown 8	619	1.11	0.05	233	121	224	141
NAS	unknown 9	427	-1.79	0.93	139	475	161	299
	unknown 10	537	-4.92**	0.5	0	69	0	47
	unknown 11(3 signal)	621	-1.6	0.59	57	191	56	196

Analysis of known compounds

In addition to untargeted data analyses, we analysed the presence and relative abundance of known metabolites. The first group of known compounds consisted of secondary metabolites that were previously identified in *B. vulgaris*: two saponins (Shinoda et al., 2002; Agerbirk et al., 2003b), glucosinolates (Agerbirk et al., 2001b; Van Leur et al., 2006) and seven flavonoids (Senatore *et al.*, 2000). The two saponins were detected in shoots (2 mass signals each) and in roots (1 mass signal; Table 3.3). Because the same isomer was detected in roots and shoots, we could compare the relative concentrations. For both saponins, the concentration in the shoots was higher than in the roots (ca. 60 times for saponin 1 and ca. 1500 times for saponin 2).

Gluconasturtiin main mass signals were saturated and therefore could not be used for reliable quantification. Analysis of its unsaturated minor mass signals revealed that, according to our expectations, gluconasturtiin was more abundant in NAS-type plants than in BAR-type plants (Table 3.3). However, this chemotypic difference was only significant for shoots and not for roots, though root gluconasturtiin levels were significantly different between the families (Table 3.3). As glucobrassicin and flavonoid 3 and 5 have the same exact mass, and their chromatograms overlapped, we used two minor mass signals (449 and 451) of glucobrassicin that were not derived from one of these flavonoids. These mass signals showed no significant differences for glucobrassicin between chemotypes or families. Neoglucobrassicin was detected in roots and shoots and varied only significantly between families. The other glucosinolates previously detected in *B. vulgaris*, 4-

methoxyglucobrassicin and 1,4-dimethoxyglucobrassicin, were not detected (Agerbirk et al., 2001b; Van Leur et al., 2006).

Table 3.3 Masses and Mann-Whitney U statistics (Z-values and significance levels: *P < 0.05, ** P < 0.001) on mass signals of LC-QTOF-MS derived known metabolites, from shoot and root tissue of two *Barbarea vulgaris* chemotypes (Type) of two families.

	Mass		Shoot		Root	
Description	**Calculated exact**	**Used for analysis**	**Type**	**Family**	**Type**	**Family**
saponin 1[a]	795.4531	795	0.53	0.63	0.76	-177.69
		841	0.67	0.38	n.d.[g]	n.d.
saponin 2[b]	779.4582	779	-0.23	303.13*	0.9	-253.60*
		826	0.03	310.86*	n.d.	n.d.
gluconasturtiin	438.0529	424	434.70**	0.92	0.36	198.39*
glucobrassicin	447.0532	449	174.69	190.31	-122.28	-0.03
		451	190.94	179.43	-139.5	0.17
neoglucobrassicin	477.0638	477	-0.31	-0.2	-170.5	196.67*
flavonoid 1	623.1612	623	214.23*	181.33	n.d.	n.d.
flavonoid 2	609.1456	609	-0.67	350.48**	n.d.	n.d.
		609	-0.94	-256.00*	n.d.	n.d.
flavonoid new 1 [c]	755.2040	755	0.36	-344.38*	n.d.	n.d.
flavonoid new 2 [d]	593,1512	595	-0.52	-283.43*	n.d.	n.d.
flavonoid new 3 [e]	933.2401	933	-0.88	0.43	n.d.	n.d.
flavonoid new 4 [f]	539.1254	539	0.52	-143.24	n.d.	n.d.
Chlorogenic acid	354.0951	353	-115.11	-157.68	146.39	-0.48
Citric acid	192.0270	191	-166.57	0.35	-0.64	251.87*
Coumaric acid	164.0473	325	-0.45	-0.57	0.02	0.79
Ferulic acid	194.0579	355	0.61	-0.57	-0.72	-0.33
Sucrose	342.2965	342	0.47	0.98	-163.61	-0.53

[a] 3-O-[O-beta-D-glucopyranosyl-(1→4)-beta-D-glucopyranosyl]-hederagenin

[b] 3-O-beta-cellobiosyloleanolic acid

[c] kaempferol-glucose-glucose-rhamnose

[d] kaempferol-glucose-rhamnose

[e] quercitine-glucose-glucose-glucose-rhamnose

[f] anthocyanin with absorption at 520 nm

[g] n.d. = not detected

Of the flavonoids described by Senatore et al. (2000) we detected flavonoids 1 and 2 in shoots. Surprisingly, flavonoid 1 was differentially expressed in the two chemotypes, with the highest expression in the NAS-type (Table 3.3). However, the mass signal areas were very low (ca. ten times lower than other flavonoids) and when each family was analyzed separately, there was only a chemotype effect within EL8 (Mann-Whitney U test: EL 8, Z = -2.27, P = 0.02; EL22, Z = -0.53, P = 0.596). As mentioned above, flavonoids 3 and 5 could not be separated from glucobrassicin and flavonoids 4, 6 and 7 were not detected in our samples. None of the flavonoids were detected in roots.

The second group of known compounds are plant metabolites, possibly involved in defence, that have never been specifically described for *B. vulgaris.* In this group we detected several flavonoids which will further be referred to as "new flavonoids". All new flavonoids were only detected in shoot tissue, and had no different levels in the chemotypes (Table 3.3).

The last group of known compounds consisted of common plant metabolites. We found mass signals representing four common acids (chlorogenic, citric, coumaric and ferulic acid) and one sugar (sucrose). All these compounds were detected in root and shoot tissue and showed no chemotype differences (Table 3.3). Only citric-acid was differentially expressed between both families. Taking together all three studied groups of known metabolites, we can conclude that only flavonoid 1 showed a chemotype effect, but this effect was inconsistent between the two families. All other tested metabolites did not co-vary with the glucosinolate types.

Seed biomass

Analysis of mixed seed batches (EL bars Figure 3.2) showed that seeds with a BAR phenotype overall had a lower mass than seeds with a NAS phenotype in the same family (Table 3.4: row 1). However, the differences between families were much larger than between chemotypes within family (Figure 3.2, Table 3.4: row 1). This strong maternal effect was confirmed by the seed batches containing only BAR-type (HE35, HE 39) or only NAS type seeds (selfings 14 and 15) (Figure 3.2; Table 3.4: row 2). These data show that, depending on the maternal plant, BAR-type seeds may weigh more or weigh less than NAS-type seeds.

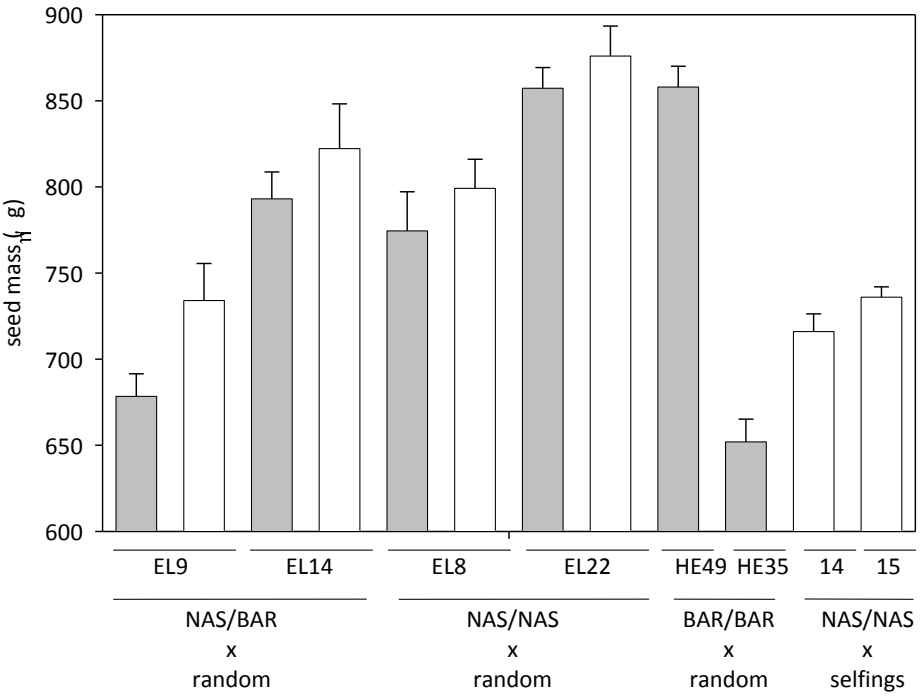


Figure 3.2 Seed mass ± SE of BAR-type (grey bars) and NAS-type (white bars) seeds, derived from half-sib families of Barbarea vulgaris. The half-sib family, maternal genotype and cross is indicated underneath the x-axis.

Germination

In none of the germination stages, the chemotypes showed a significantly different germination rate (Table 3.4: rows 3-6). However, germination rate significantly differed between families in the early phases (bursted seed coat, root development and unfolding of cotyledons; Table 3.4: rows 3-5). Eventually, the final germination stage was similar for all seeds (time until leaf primordium formation; Table 3.4: row 6). Of the 349 seeds 98.6% developed the leaf primordium of the first true leaves within 11 days and were considered as successfully germinated. As for all chemotype-family combinations germination was higher than 95%, we conclude that neither chemotype nor family had a considerably negative effect on germination success.

Table 3.4 F-values (or Z-value) and significance levels (*$P < 0.05$, ** $P < 0.001$) of all growth and development parameters tested on the two *Barbarea vulgaris* chemotypes (Type) on several Families (Family) and on time points (Day).

	Parameter	Type	Family	Day (#)	T × F	T × D	F × D	T × D × F
Seed mass								
1	mixed batches	$F_{1,341} = 5.76$*	$F_{3,341} = 27.63$**	n.a.[a]	0.40	n.a.	n.a.	n.a.
2	pure batches	$Z_{1,16} = 2.61$*	$F_{3,16} = 64.52$**	n.a.	n.a.	n.a.	n.a.	n.a.
Germination (days until stage is reached)								
3	seed coat	$F_{1,341} = 0.68$	$F_{3,341} = 2.63$*	n.a.	0.81	n.a.	n.a.	n.a.
4	root	$F_{1,341} = 0.00$	$F_{3,341} = 4.44$*	n.a.	0.90	n.a.	n.a.	n.a.
5	cotyledon	$F_{1,341} = 0.71$	$F_{3,341} = 5.80$**	n.a.	0.40	n.a.	n.a.	n.a.
6	primordium	$F_{1,341} = 1.04$	$F_{3,341} = 0.49$	n.a.	0.53	n.a.	n.a.	n.a.
Seedling								
7	root length	$F_{1,142} = 0.92$	$F_{1,142} = 18.97$**	n.a.	6.53*	n.a.	n.a.	n.a.
8	shoot length	$F_{1,142} = 0.00$	$F_{1,142} = 98.79$**	n.a.	0.31	n.a.	n.a.	n.a.
9	cotyledon width	$F_{1,142} = 0.11$	$F_{1,142} = 56.36$**	n.a.	0.59	n.a.	n.a.	n.a.
10	cotyledon length	$F_{1,142} = 0.45$	$F_{1,142} = 13.35$**	n.a.	0.71	n.a.	n.a.	n.a.
Dry mass								
11	root	$F_{1,160} = 0.04$	$F_{1,160} = 8.39$*	$F_{4,160} = 139.54$**	0.06	1.26	8.06**	0.58
12	shoot	$F_{1,160} = 0.13$	$F_{1,160} = 0.71$	$F_{4,160} = 176.31$**	0.15	0.76	1.87	0.15
13	total	$F_{1,160} = 0.41$	$F_{1,160} = 5.45$*	$F_{4,160} = 264.65$**	0.02	1.63	7.84**	0.34
14	root/shoot ratio	$F_{1,160} = 0.02$	$F_{1,160} = 0.02$	$F_{4,160} = 27.66$**	0.91	0.05	1.45	1.58
Leaf characteristics								
15	area	$F_{1,26} = 0.60$	$F_{1,26} = 1.83$	n.a.	0.88	n.a.	n.a.	n.a.
16	# leaves	$F_{1,74} = 2.01$	$F_{1,74} = 43.57$**	$F_{5,74} = 1303.83$**	0.27	1.60	13.04**	0.37
17	maximum length	$F_{1,74} = 3.13$	$F_{1,74} = 8.29$*	$F_{4,74} = 616.25$**	0.06	1.84	8.32**	0.1
18	# leaf lobs	$F_{1,73} = 0.48$	$F_{1,73} = 5.26$*	$F_{4,73} = 337.39$**	0.01	0.31	13.36**	0.73
Flower stalks								
19	# flower stalks	$F_{1,53} = 3.21$	$F_{1,53} = 5.31$*	n.a.	0.59	n.a.	n.a.	n.a.
20	total length	$F_{1,53} = 2.41$	$F_{1,53} = 13.86$**	n.a.	0.78	n.a.	n.a.	n.a.

[a] n.a. = not applicable

Growth experiment

Despite lower seed masses, the root and shoot length as well as the cotyledon width and length of 16-day-old seedlings of EL8 were larger than those of EL22 (Figure 3.3; Table 3.4: rows 7-10). However, there was no significant effect of chemotype on these seedling characteristics (Table 3.4: rows 7-10). For seedling root length, there was a slightly significant (P = 0.044) interaction between type and family. Plant biomass (root, shoot and total) increased over time and was only significantly affected by family and not by chemotype (Figure 3.4; Table 3.4: rows 11-13). In contrast to the biomass, the root/shoot ratio was not significantly affected by family, but only changed over time (Table 3.4: row 14).

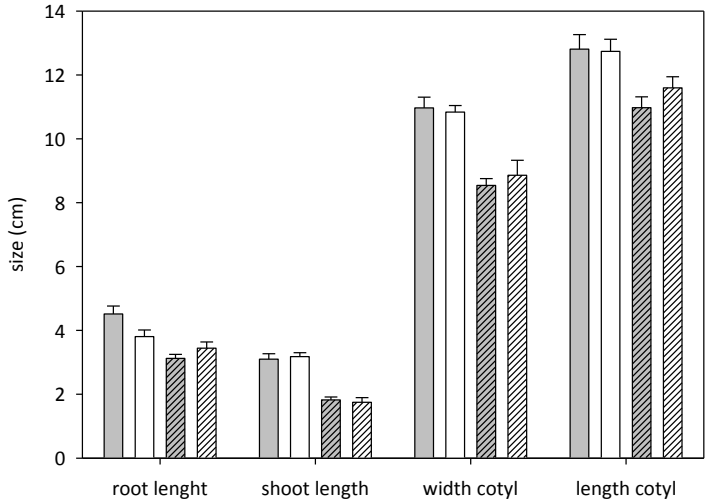


Figure 3.3 Root and shoot length and cotyledon width and length of 16-day-old BAR-type (grey bars) and NAS-type (white bars) Barbarea vulgaris seedlings of half-sib family EL8 (open bars) and EL22 (hatched bars).

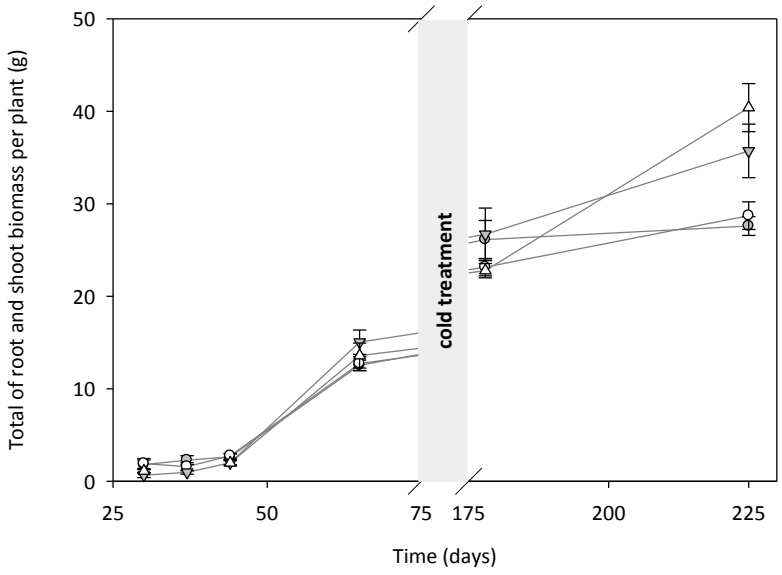


Figure 3.4 Time series of total dry mass (root + shoot) of *Barbarea vulgaris* BAR-type (grey) and NAS-type (white) plants of half-sib family EL8 (circles) and EL22 (triangles). The insert represents a vernalization period to induce flowering.

At several time points leaf characteristics were measured. The number of leaves, length of the longest leaf and the number of leaf lobes were not different for the two chemotypes, but only differed between families, and there was a family × time (Day) interaction (Table 3.4: rows 16-18). The leaf area at day 44 was not affected by type or family (Table 3.4: row 15).

After vernalization all plants started to flower. Plants from the EL8 family produced more flower stalks, and flower stalks had higher total length than those from EL22 (Figure 3.5, Table 3.4: rows 19-20). Despite a slightly larger number of flower stalks in BAR-type plants of both families, this effect was not statistically significant (Figure 3.5, Table 3.4: rows 19-20).

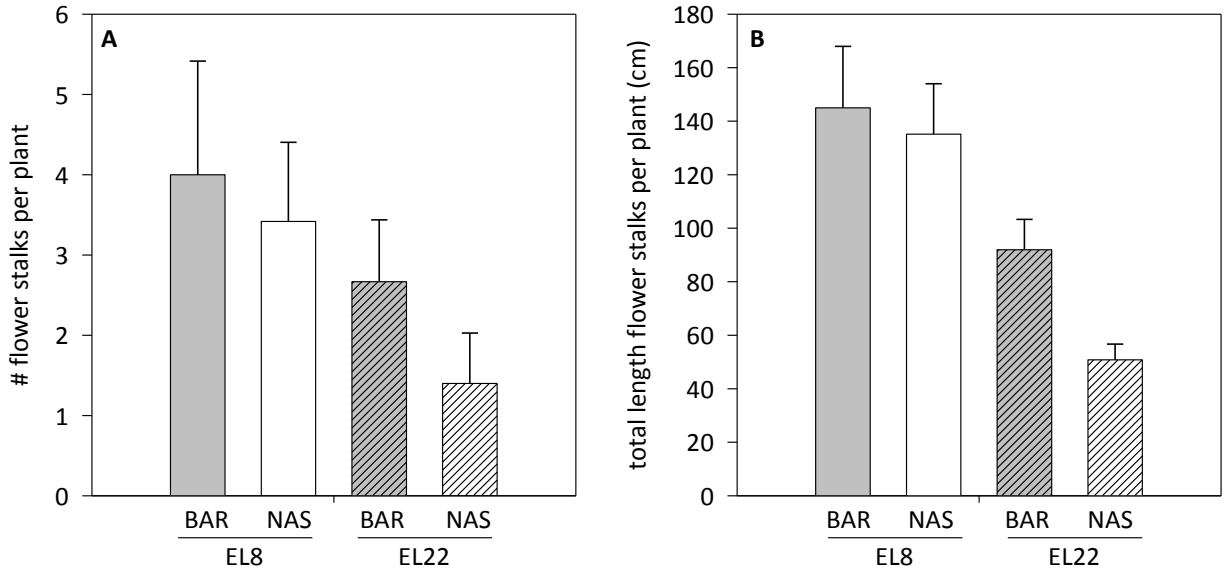


Figure 3.5 Number of flower stalks (A) and total length of flower stalks (B) of BAR-type (grey bars) and NAS-type (white bars) *Barbarea vulgaris* seedlings of half-sib family EL8 (open bars) and EL22 (hatched bars).

Discussion

Metabolomics

The metabolite analyses showed that ca. 92%, of the mass signals, that contributed highly to the discrimination of BAR-type and NAS-type plants in both families corresponded to glucosinolates. Additionally, three BAR/SIB related mass signals significantly differed between chemotypes. The rest of the chemotype differentiating signals could not be identified yet (Table 3.3). For further identification, additional LC-MS/MS fragmentation experiments would be needed. Because the 71 mass signals that were omitted from the analyses due to detector saturation, were derived from glucosinolates, the contribution of glucosinolates to separation of BAR-type and NAS-type samples probably is larger than shown in these analyses.

Of the previously reported secondary metabolites in *B. vulgaris*, none was consistently different between chemotypes (Table 3.4). Flavonoid 1 (Senatore *et al.*, 2000) showed an overall significant chemotype effect in shoot samples, but more detailed analysis showed that this difference was only present in one family. More half-sib families need to be analysed before we can conclude that this difference is unique to this family. In addition to the flavonoids reported in earlier studies, we were able to identify several flavonoids and phenolic compounds that were not previously reported for *B. vulgaris*.

Chlorogenic acid, ferulic acid and coumaric acid, as well as gluconasturtiin and glucobarbarin are phenylalanine derived metabolites. As they use the same substrate, up-regulation of one of the pathways could, due to substrate limitation, result in down regulation of the other compounds. If this were the case, BAR type plants with higher levels of phenylalanine-derived glucosinolates may have shown lower levels of phenolic acids. Our analysis, however, showed no differences in phenolic acids levels between chemotypes. Hence, we found no evidence for trade-offs between these pathways.

In general, this metabolome-wide analysis confirmed our earlier findings using targeted HPLC glucosinolate analyses. The main difference between the chemotypes is that BAR-type plants contain more glucobarbarin and NAS-type plants more gluconasturtiin. The metabolomic analyses also show that the chemotypes differ more in shoot than in root chemistry (Van Leur et al., 2006). Even though glucobarbarin is significantly more abundant in BAR-type roots than in NAS-type roots, the root gluconasturtiin levels did not differ significantly between chemotypes. In roots we also found fewer discriminating metabolites than in shoots, and the metabolites differed less significantly between the chemotypes. We therefore expect the chemotypes to have a more clearly differential effect on shoot herbivores than on root herbivores (Chapter 6).

Growth and development

Despite profound differences in the glucosinolate profiles and levels, the chemotypes did not differ in growth rates. Besides the higher seed mass of BAR-type seeds, there were no differences in *in vitro* germination, biomass accumulation, leaf characteristics and flowering phenology. Moreover, the chemotype effect on seed mass was smaller than the variation between the families. Therefore, we conclude that there are no direct costs for growth and development that would result into differential selection for either chemotype.

Variation between half-sib families

For metabolites as well as seed, seedling, rosette and flowering plants, we found significant variation between half-sib families. These differences can be due to maternal effects. Seed mass, for example, may greatly depend on the nutritional status of the mother plant. Large and resource rich mother plants are likely to produce seeds with a higher seed mass (Fenner, 1991). It has been suggested that the glucosinolates in seeds are not only derived from *de novo* biosynthesis in silique walls, but also from long-distance transport from the maternal plant (Du and Halkier, 1998). Consequently, resource allocation costs in seeds and seedlings might be diminished by allocation of glucosinolates from the parent plant into the seed.

However, van Leur et al (2006) showed that for *B. vulgaris* seeds, the glucosinolate type may differ from that of the maternal phenotype, and hence is determined by its own genotype. The observed differences in seed mass between BAR-type and NAS-type plants could therefore be caused by differences in glucosinolate profiles.

Next to seed mass, the families differed in early indicators of germination rate. However, for the final germination stage (primordium formation) there was no significant family effect. As the seedlings only had sterilised water and glass beads to germinate on, nutrient deficiency in fast germinating seeds may have slowed their development, hence reducing the later differences.

In general, the fitness effect of any gene depends in a complicated way on many other genes. This interaction between genes (also called epistasis) may have differed between half-sib families due to differences in genetic background. As the genetic background of half-sibs has a high overlap for maternal genes, epistatic effects can be similar within a half-sib family, but differ between half-sib families. This might explain the variation between the half-sib families.

Conclusions

The *B. vulgaris* glucosinolate type did not show large differences in metabolite content, other than glucosinolates and there were no differences in growth and development. We therefore conclude that there are no, or hardly any direct costs associated to being one, or another chemotype. Our findings also indicate that differential effects of the chemotypes on different herbivores (Chapters 4, 5 and 6) are truly due to the differences in glucosinolate profiles and not to other compounds or differences in growth and development. Except for seed mass analysis, we only compared heterozygous plants with homozygous recessive plants. It remains possible that homozygous BAR-type plants do show direct costs. However, the absence of intermediate glucosinolate levels in heterozygous genotypes suggests that the BAR-allele is fully dominant over the NAS-allele, which makes the presence of phenotypic differences between homozygous and heterozygous BAR-type plants unlikely. Based on the observed absence of direct costs, we expect that the existence and maintenance of the glucosinolate polymorphism in *B. vulgaris* is more likely due to different frequencies of herbivores in the plant populations, than by direct metabolic costs.

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CHAPTER 4

Barbarea vulgaris glucosinolate phenotypes differentially affect performance and preference of two different species of lepidopteran herbivores

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Abstract

The composition of secondary metabolites and the nutritional value of a plant both determine herbivore performance and preference. The genetically determined glucosinolate pattern of *Barbarea vulgaris* can be dominated by either glucobarbarin (BAR-type) or by gluconasturtiin (NAS-type). Because of the structural differences, these glucosinolates may have different effects on herbivores. We compared the two *Barbarea* chemotypes with regards to the preference and performance of two lepidopteran herbivores, using *Mamestra brassicae* as a generalist and *Pieris rapae* as a specialist. The generalist and specialist herbivores did not prefer either chemotype for oviposition. However, the larvae of the generalist *M. brassicae* preferred to feed and performed best on NAS-type plants. On NAS-type plants 100% of the *M. brassicae* larvae survived while growing exponentially, whereas on BAR-type plants *M. brassicae* larvae showed little growth and a mortality of 37.5%. In contrast to *M. brassicae,* the larval preference and performance of the specialist *P. rapae* was unaffected by plant chemotype. Total levels of glucosinolates, water soluble sugars and amino acids of *B. vulgaris* could not explain the poor preference and performance of *M. brassicae* on BAR-type plants. Our results suggest that the difference in glucosinolate chemical structure is responsible for the differential effects of the *B. vulgaris* chemotypes on the generalist herbivore.

Keywords

Glucosinolates, Oviposition, Performance, Polymorphism, *Barbarea vulgaris, Pieris rapae, Mamestra brassicae*, Co-evolution.

Introduction

Glucosinolates and their breakdown products are involved in plant defence against a wide variety of potential plant enemies. On the other hand, they can also serve as feeding and oviposition stimulants for specialist herbivores (Chew, 1988; Louda and Mole, 1991; Wittstock et al., 2003). The composition of glucosinolates is genetically variable within plant species, and strongly influences the feeding choices of insect herbivores (Kroymann et al., 2001; Lambrix et al., 2001). A heritable glucosinolate polymorphism in *Barbarea vulgaris* results in two discrete chemotypes (Van Leur et al., 2006). In this paper we investigate the effect of these two chemotypes on the preference and performance of two lepidopteran herbivores.

The most common and genetically dominant chemotype of *B. vulgaris* forms mainly (*S*)-2-hydroxy-2-phenylethyl-glucosinolate (glucobarbarin, BAR-type). Although depending on the presence of cofactors like ESP, pH or metal ions (Burow et al., 2006), the most likely initial hydrolysis product of glucobarbarin is an isothiocyanate, which due to the 2-hydroxylation of the glucosinolate side chain spontaneously cyclizes to 5-phenyloxazolidine-2-thione (Kjaer and Gmelin, 1957). This glucosinolate breakdown product is known to reduce infection by the soil fungus *Plasmodiophora brassicae* (Ludwig-Müller et al., 1999). Hardly anything is known about other ecological activities of oxazolidine-2-thiones (Wittstock et al., 2003).

The less abundant and genetically recessive chemotype of *B. vulgaris* contains mainly 2-phenylethyl glucosinolate (gluconasturtiin, NAS-type). 2-Phenylethyl glucosinolate is also present in *Arabidopsis thaliana* and other *Brassicaceae* (Reichelt et al., 2002), predominantly in root tissues (Sang et al., 1984). Its most likely breakdown product is 2-phenylethyl isothiocyanate. Isothiocyanates are the predominant breakdown products of glucosinolates and are generally toxic to various herbivores (Wittstock et al., 2003). 2-Phenylethyl isothiocyanate negatively affects a very broad range of phytophages, e.g., nematodes (Potter et al., 1999; Potter et al., 2000; Serra et al., 2002; Lazzeri et al., 2004), snails (Kerfoot et al., 1998), flies, aphids, mites (Lichtenstein et al., 1962), fungi (Sarwar and Kirkegaard, 1998) and several generalist and specialist Lepidoptera (Wadleigh and Yu, 1988; Borek et al., 1998). Despite counter-adaptations of specialists to reduce or circumvent negative effects of glucosinolates (Ratzka et al., 2002; Wittstock et al., 2003), isothiocyanates can still reduce survival and growth, and increase development time of specialists (Agrawal and Kurashige, 2003). In contrast to the oxazolidine-2-thiones formed in BAR-type plants, which can increase the incidence of goiter in mammals, the 2-phenylethyl isothiocyanate formed in NAS-type plants has chemopreventive effects against tumorigenesis in mammalian organisms (Musk et al., 1995; Griffiths et al., 1998; Canistro et al., 2004a).

Based on the specific biological effects of the expected breakdown products of glucobarbarin and gluconasturtiin we hypothesize that NAS-type and BAR-type *B. vulgaris* plants have differential effects on insect herbivores. Generally it is expected that chemical plant defences are more effective against generalist herbivores than against specialists (Cornell and Hawkins, 2003). Therefore we compared the preference and performance on the two chemotypes of a generalist (*Mamestra brassicae*) and a specialist (*Pieris rapae)* herbivore which are both well-studied, important crucifer pests (Theunissen et al., 1985; Finch and Kienegger, 1997).

M. brassicae is a generalist feeding on plants in 70 species and 22 families, of which Brassicaceae are among the most preferred (Rojas et al., 2000). Even though *M. brassicae* can detect glucosinolates by receptor cells on the sensilla (Wieczorek, 1976) and its oviposition is stimulated by damaged cabbage plants (Rojas, 1999), no physiological adaptations of this species to glucosinolates have been described yet. Larvae of *Pieris rapae,* on the other hand, can detoxify glucosinolates by shifting hydrolysis products from isothiocyanates to less toxic nitriles using the myrosinase directing nitrile-specifier protein (NSP). This enables *P. rapae* to consume foliage that is otherwise well defended (Wittstock et al., 2003; Wittstock et al., 2004). Female adults of *Pieris rapae* can detect intact glucosinolates in leaves of Brassicaceae with specialized receptor cells and are stimulated to lay eggs on glucosinolate containing plants.

We assessed herbivore preference on the level of adult oviposition and larval feeding. The female oviposition preference initially determines the host of the larvae (Akhtar and Isman, 2003). As the isothiocyanates from NAS-type plants are expected to be more toxic than the oxazolidine-2-thiones produced by BAR-type plants, we expect *M. brassicae* to be repelled by NAS-type plants and thus to prefer BAR-type plants for oviposition. Oviposition of *P. rapae* is affected by glucosinolates at the leaf surface (Renwick et al., 1992; Van Loon et al., 1992) and known to be stimulated by glucobarbarin as well as by gluconasturtiin in a dose-dependent way (Chew, 1988; Huang and Renwick, 1994; Huang et al., 1994).

As larvae can eventually leave their initial host (Van Dam et al., 2000), we also assessed larval preference. If the *B. vulgaris* chemotypes differ in toxicity or palatability we expect to see the largest effect on larval preference and larval performance for the unadapted generalist *M. brassicae* and no or minor effects on the glucosinolate specialist *P. rapae*.

Although we observed no differences in morphology, growth or germination between the chemotypes (Chapter 3), pleiotropic effects or close linkages can possibly cause other genes and metabolites to be consistently different between the chemotypes. As the nutritional value of the plants is important for herbivore performance and preference (Slansky and Rodriguez, 1987; Simpson and Simpson, 1990; Berenbaum, 1995), we additionally analyzed the sugar content and amino acid level of the tissue on which the herbivores were feeding.

Methods and materials

Plant material

B. vulgaris seeds were collected from 10 individual BAR and their nearest neighbour NAS-type maternal plants, which were freely cross pollinated in a natural population of *B. vulgaris*. The population was located in Elderveld, The Netherlands (51.95 °N; 5.87 °E) and consisted of 22% NAS-type plants (Van Leur et al., 2006). We selected offspring of maternal NAS-type plants "EL44" (68% BAR-type offspring) and "EL13" (62% BAR-type offspring). Plants were grown in a glasshouse, at 21 °C (day) and 16 °C (night), with 60% relative humidity and natural daylight supplemented with sodium lamps to maintain the minimum PAR at 225 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a photoperiod of 16:8 (L:D). One week after germination on glass beads, the seedlings were transplanted to a mixture of peat soil (Potgrond 4, Lentse Potgrond BV., Lent, The Netherlands) and 20% sand. After 2 weeks the seedlings were transplanted to 1.1L pots, watered and fertilized regularly with half strength Hoagland's nutrient solution with a doubled KH_2PO_4 content.

Chemical analysis

For quantification of glucosinolates, soluble sugars and amino acids, one global extraction was used. In a 2 ml Eppendorf tube 50.0 mg of lyophilized finely ground plant material was dissolved in 1.0 ml 70% MeOH in water (v/v), vortexed and immediately boiled for 5 min. to kill remaining myrosinase activity. Tubes were placed in an ultrasonic bath for 15 min. and centrifuged (10 min. 10000 r.p.m). The extraction was repeated for the pellet omitting the boiling step. For each sample, both supernatants were combined in a new 2 ml Eppendorf tube and supplemented individually with 70% MeOH to attain the average mass (n = 3) of a 2 ml Eppendorf tube containing 2.0 ml 70% MeOH. This "stock" extract was stored at -20 °C until further analysis.

Half (1.0 ml) of the stock extract was used for glucosinolate analysis and applied to a DEAE-Sephadex A 25 column (EC, 1990), desulphated with arylsulphatase (Sigma, St. Louis, IL, USA) and separated on a reversed phase C-18 column on HPLC with a $CH_3CN–H_2O$ gradient as described in van Dam et al. (2004). Glucosinolate analysis was performed with a PDA detector (200–350 nm) with 229 nm as the integration wavelength. Desulfoglucosinolate peaks were identified by comparison of HPLC retention times and UV spectra with authentic standards isolated from *B. vulgaris* as previously described (Agerbirk et al., 2001b), as well as standards kindly provided by M. Reichelt, MPI Chemical Ecology, and a certified rape seed standard (Community Bureau of Reference, Brussels, code BCR-367R). The same response factor was used for glucobarbarin as for gluconasturtiin (Buchner, 1987). To calculate glucosinolate concentrations in the plant tissue, the obtained values were multiplied by two and divided by dry mass.

To analyze soluble sugar content, a 10 µl aliquot of the stock extract was diluted with 990 µl MilliQ water. Soluble sugars were analyzed by injecting 5 µl of the diluted extract on Dionex HPLC system, equipped with a Carbopac PA1 column (2 × 250 mm) and a Carbopac PA1 guard column (2 × 50 mm, Dionex Corp. Sunnyvale CA, USA). An isocratic gradient mixture of 10% 1 M NaOH and 90% MilliQ water was used to separate the sugars at a flow rate of 0.25 ml/min. Column temperature was kept at 20 °C. A "10 ppm" reference solution containing

54.9 µM sorbitol and manitol, 29.21 µM trehalose, sucrose and melbiose, and 55.51 µM glucose and fructose, was diluted to obtain 7.5 ppm, 5 ppm and 2.5 ppm calibration standards to obtain a reference curve. After every 10 samples an additional standard was injected to check for deviations of retention times and the calibration curve. To calculate the molar concentration of sugars in the plant tissue, the concentration values were multiplied by 200 and divided by dry mass.

Amino acids were analyzed on a Dionex HPLC system by integrated pulsed amperometric detection. An aliquot of 20 µl of the stock extract was diluted with 980 µl MilliQ. Of this diluted extract 25 µl was injected and amino acids were separated with a ternary gradient (see DIONEX application update 152, Method 1, standard AAA gradient; condition 60/2 in Hanko and Rohrer, 2004) on a 2 × 250 mm AminoPac© PA10 column with a 2 × 50 mm AminoPac© PA10 Guard column (Dionex, Sunnyvale,CA, USA). Eluents, flow rates, waveform and working electrode conditions were all as specified under Method 1 in Dionex application update 152 and in Hanko and Rohrer (2004). The Sigma AA-S-18 amino acid standard (Sigma, St Louis, MO, USA) containing 17 amino acids was supplemented with asparagine, glutamine and tryptophane (2.5 µmoles/ml each) to obtain a reference sample containing the 20 most common amino acids. This reference solution was diluted to obtain calibration standard ranging from 1 – 8 µM for each amino acid, except for cysteine, which had a range of 0.5 – 4 µM. After every 10 samples an additional standard was injected to check for deviations of retention times and the calibration curve. To calculate the molar concentration of the amino acids in the plant tissue, the concentration values were multiplied by 200 and divided by dry mass.

To determine the chemotype of each plant, glucosinolates were extracted from the first full grown leaf and analysed on HPLC as described above. When the peak area of glucobarbarin divided by the peak area of gluconasturtiin was > 10, the plant was considered a BAR-type, when this ratio was < 0.1 it was considered a NAS-type.

Oviposition

Insects were obtained from the Laboratory of Entomology of Wageningen University the Netherlands. Stock colonies of *Pieris rapae* and *Mamestra brassicae* were maintained on *Brassica oleracea* var. *gemnifera* L., cultivar Cyrus, in a climatized room at 20-22 °C, 50-70% relative humidity and a photoperiod of 16:8 (L:D). We used ca. four month old half-sib plants of family EL44 which were clipped one month before use to ensure abundant fresh leaf material. At the start of the experiment plants were moved from the glasshouse to a climatized room at 21 °C (day) 16 °C (night), 60% relative humidity, illuminated to 200 PAR at plant height (Philips Master TLD 50W/840 HF and 60W lights) and a photoperiod of 16:8 (L:D). In the same room, one to three days old adult insects were held in a mesh cage (40 × 45 × 65 cm^3) provided with sugar solution. Males and females were held together to mate for at least 24 hours. Oviposition preference was assessed by introducing individual pairs into one of the 11 oviposition mesh cages (40 × 45 × 65 cm^3) each containing 1 BAR-type and 1 NAS-type plant and a source of sugar. Oviposition was checked every day at 10.30h and 15.30h. *M. brassicae* oviposition was recorded for min. 24h after the first egg up to seven days after introduction (n = 68). *P. rapae* oviposition was recorded for at least 5h after the first egg and max. 24hr in total (n = 35). Eggs deposited on the cage, pot or on the label were

not included in the analyses. To obtain sufficient replicates, four randomly chosen plants were used twice for *M. brassicae*, but oviposition preference was never tested on the same combination of plants.

Larval preference

For *P. rapae* as well as for *M. brassicae* we used plants from EL44 and EL13 half sibs (1 plant per chemotype per half-sib family and per herbivore). Two 0.9 cm diameter discs of each chemotype were cut from fully expanded leaves and placed in a circle (in an alternated design) in a 12 cm diameter Petri dish. We tested neonate larvae and five days old larvae which, until use, were feeding on *Brassica oleracea* var. *gemnifera* L., cultivar Cyrus leaves. The larvae were released individually at the centre of the Petri dish with equal distance to all leaf discs (n = 20 per species per age). After 4 hr, the amount of leaf material consumed was recorded visually and categorized as follows: 0 = no damage, 1 = only consumption of the abaxial leaf layers, 2 = less then 10% was consumed, 3 = between 10% and 50% of the leaf disc was consumed, and 4 = more than 50% was consumed. We were not able to assign consumption categories in the experiment with neonates because they caused so little damage, but five days old larvae showed distinct feeding patterns.

Larval performance

Larval performance was tested by forcing neonate larvae to stay on selected plants and measuring larval biomass and survival every other day. We selected 40 six week old plants (20 EL44 half sibs and 20 EL13 half sibs; 10 of each chemotype per half sib family). To obtain a total of 80 plants, every plant was multiplied by cutting it into two halves and growing each plant in fresh pots. After three weeks, the plants were used to test performance of *M. brassicae.* Larvae were kept on the plant using 25 mm diameter meshed clip cages. After four days these clip cages were replaced by 55 mm diameter meshed clip cages. After eight days, *M. brassicae* larvae had consumed such large amounts of leaf tissue that the experiment had to be stopped. The remaining larvae were removed and weighed. All plants were clipped and after three weeks of regrowth used to asses performance of *Pieris* in the same way as we did for *M. brassicae*. However, *P. rapae* larvae were kept on the plants till day 18 when most larvae had pupated. Until day four each larva was feeding on a single leaf which we harvested to measure the consumed leaf area and sugar, glucosinolate and amino acid content. These samples were considered to provide an estimate for the chemotype differences over the first eight days of feeding.

The long term performance of *M. brassicae* larvae was studied in a second experiment, in which three neonate larvae were placed on one plant (15 BAR and 15 NAS-type plants of EL 13). The larvae could freely move on the plant, but could not move to other plants due to a plastic cylinder and a water barrier around each plant. After 8 and 13 days we determined larval mass and the number of dead or lost larvae. After 13 days on BAR-type plants, all but 5 larvae had died or were lost, so that we stopped the experiment. As dead larvae had often dried out, we excluded their biomass from all calculations.

Statistical analysis

In the oviposition preference experiments total egg load may differ between individuals. Therefore the number of eggs on each chemotype per individual female was treated as a paired sample. Per paired sample we considered the chemotype with the highest egg load as the preferred type. Overall preference was tested with a Sign test. The number of eggs·plant^{-1} and number of clusters·plant^{-1} were not normally distributed and analyzed with the Wilcoxon matched-pair signed-ranks test. As there were no paired observations for cluster size, these data were analyzed with the Mann-Whitney *U* test.

To analyze larval preference, we compared the average food consumption category between the two chemotypes using the Wilcoxon matched-pair signed-ranks test. In the larval performance experiments the larval masses were not normally distributed and therefore analyzed for differences between the chemotypes with separate Mann-Whitney *U*-tests for each day followed by Bonferroni correction for multiple comparisons (for *M. brassicae* $\alpha = 0.05/4 = 0.0125$; for *P. rapae* $\alpha = 0.05/8 = 0.00625$). Data on the leaf consumption of each herbivore on the two chemotypes were also tested with the Mann-Withney *U*-test. The relationships between larval biomass and leaf consumption and between larval biomass and total glucosinolate concentration were tested using Pearson product-moment-correlations. The data were log-transformed to get a normal distribution. Prior to analysis of variance (ANOVA) total glucosinolate, sugar and amino acid content data were log-transformed to meet the assumptions of ANOVA. Statistical analyses were performed with STATISTICA (data analysis software system), version 7.1. (StatSoft, Inc. (2005). www.statsoft.com).

Results

Oviposition preference

The generalist and specialist adult herbivores did not prefer one chemotype over the other. Of the 68 *M. brassicae* females tested, 33.8% oviposited on the plants, 47.1% preferred to oviposit on the cage, whereas 19.1% did not oviposit at all within 1 week. The chemotypes did not receive significantly different number of eggs (Table 4.1). *M. brassicae,* which is a gregarious species, deposited a total of 19 clusters on BAR-type and 19 clusters on NAS-type plants. The number of clusters·plant^{-1} and the average cluster size, ranging from 6 to 465 eggs·cluster^{-1}, were not significantly different between the chemotypes (Table 4.1).

Table 4.1 Oviposition of *Mamestra brassicae* and *Pieris rapae* on the BAR-type and NAS-type Barbarea vulgaris.

	Mamestra brassicae				***Pieris rapae***			
	BAR	NAS	*P*	Z	BAR	NAS	*P*	*Z*
Preference [1]	14	11	0.838	0.204	12	19	0.472	0.720
Eggs[2]	220 ± 40.9	193 ± 51.7	0.399	0.843	26.9 ± 18.2	26.6 ± 14.5	0.922	0.100
Clusters/plant[3]	1.36 ± 0.2	1.73 ± 0.3	0.951	0.061				
Cluster size[4]	162 ± 23.4	112 ± 32.9	0.800	0.254				

[1] Times when that chemotype received most eggs in a pairwise comparison
[2] Average number of eggs·plant^{-1} ± SE
[3] Average number of clusters·plant^{-1} ± SE
[4] Average number of eggs·plant^{-1} ± SE

Of the 35 *P. rapae* pairs tested, 32 females oviposited on the plants, two females did not oviposit at all and one female died. With an average of 53.5 ± 5.1 eggs per female, 61% of the females deposited most eggs on NAS-type plants and one female deposited an equal amount of eggs on both chemotypes. The average numbers of eggs deposited on each chemotype did not significantly differ between the chemotypes (Table 4.1). In total BAR-type plants received 861 eggs in and NAS-type plants 850 (Table 4.1).

Larval preference

Of the 20 five day old *M. Brassicae* larvae tested, only one preferred to feed on BAR-type leaf discs, whereas 19 preferred the NAS-type (Sign test: $Z = 3.67$, $P < 0.001$). This preference for NAS-type leaf discs was confirmed when corrected for the consumed quantity (feeding categories). *M. brassicae* larvae consumed more from NAS-type leaf discs than from BAR type leaf discs (Figure4.1; Wilcoxon matched-pairs test on feeding categories: *M. brassicae* n = 20, $Z = 3.743$, $P < 0.001$). In contrast, the 20 five day old *P. rapae* larvae did not show a significant preference. Five larvae preferred NAS-type, 11 BAR-type and four larvae consumed from BAR-type as well as NAS-type leaf discs (Sign test: $Z = 1.25$, $P = 0.211$). Although *P. rapae* larvae consumed more from BAR-type leaves the difference in feeding categories was not significant (Fig 4.1; Wilcoxon matched-pairs test on feeding categories: *P. rapae* n = *20,* $Z = 1.629$, $P = 0.103$). Consequently we found no significant preference of *P. rapae* larvae for either chemotype.

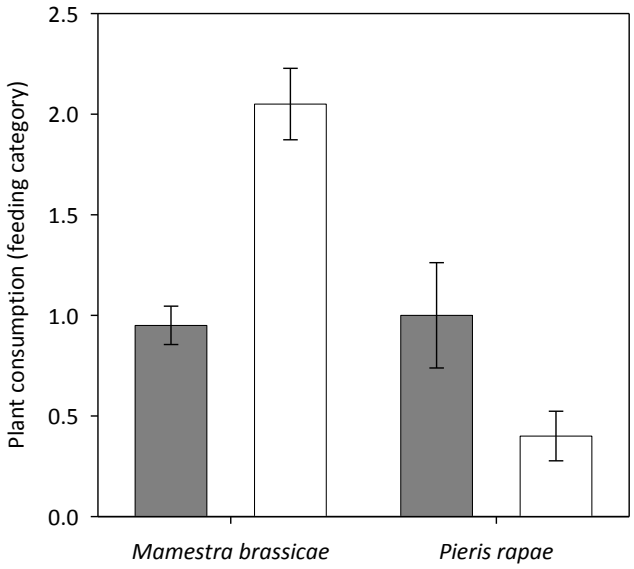

Figure 4.1 Consumption of BAR-type (grey bars) and NAS-type (white bars) leaf discs by 5 days old larvae of *Mamestra brasscicae* and *Pieris rapae* (feeding category averaged per Petridish +/- SE).

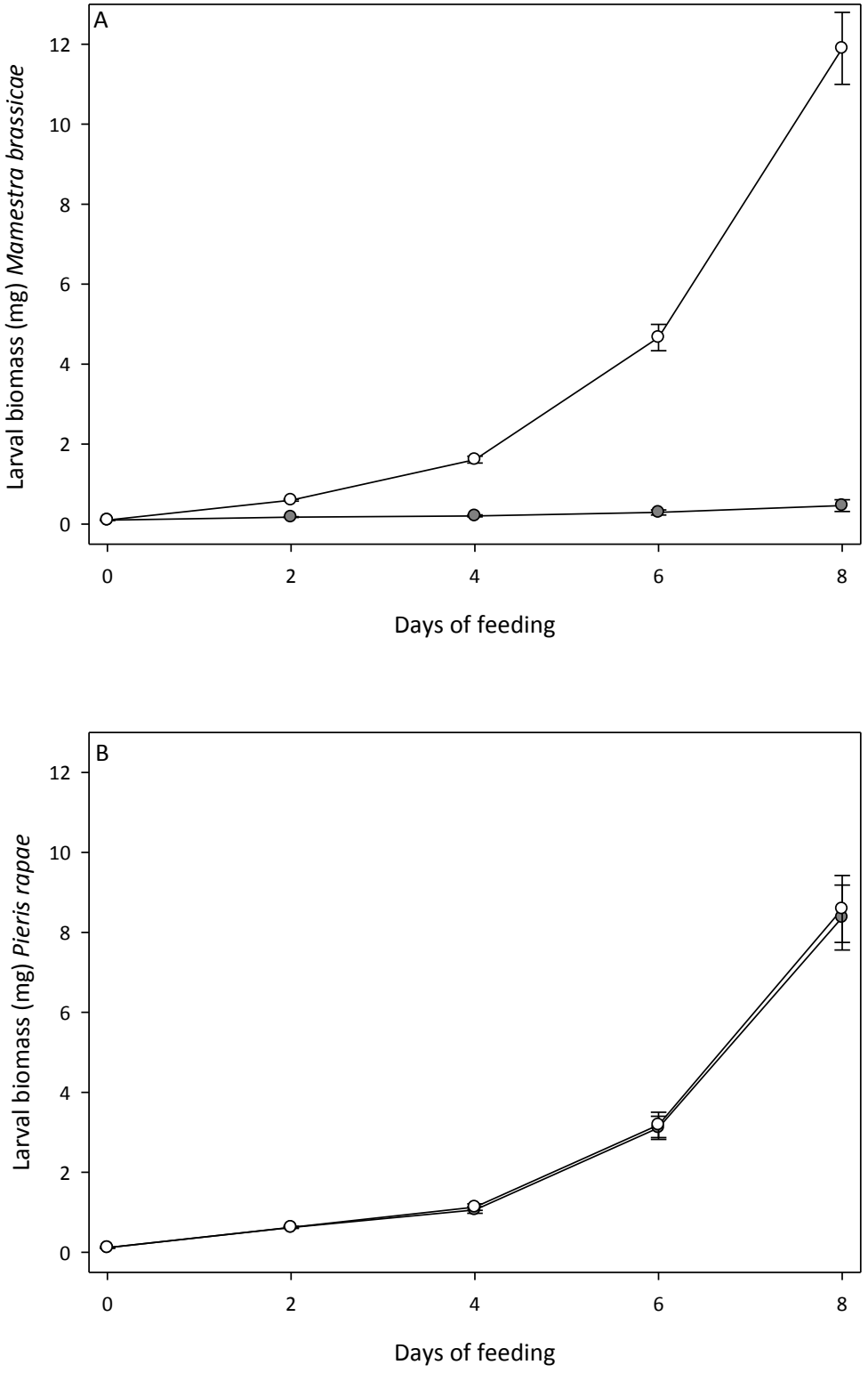

Figure 4.2 Biomass accumulation of (A) *Mamestra brassicae* and (B) *Pieris rapae* larvae during 8 days of feeding BAR-type (grey dots) and NAS-type (white dots) *Barbarea vulgaris* plants +/-SE.

Larval Performance and chemotypes

Over the first eight days, the larval biomass accumulation of the generalist *M. brassicae* was significantly affected by chemotype (Mann-Withney *U* test: $P < 0.001$ for each day of recording). When forced to stay on NAS-type plants *M. brassicae* larvae grew exponentially (Figure 4.2A). On BAR-type plants, however, *M. brassicae* larvae hardly increased in biomass and were moribund. This difference in larval performance was significantly and positively correlated to the difference in leaf consumption after four days of feeding (Correlation analysis: $r = 0.942$, $r^2 = 0.887$, $P < 0.01$). Consequently *M. brassicae* larvae caused significantly more damage to NAS-type leaves than to BAR-type leaves (Figure 4.3A; Mann-Whitney *U* test: $U = 12$, $Z = -7.583$, $P < 0.001$). Larval survival after eight days on the plants was in line with these results. On NAS-type plants all larvae were still alive, whereas on BAR-type plants 37.5% of the *M. brassicae* larvae had died. The results of the second experiment, in which the larvae could move freely on the plants, confirmed the results of the clip cage experiment. After 13 days, 89% ± 10 of the larvae had stayed and survived on NAS-type plants and weighed 42.13 ± 2.69 mg, whereas only 20% ± 10 of the larvae had stayed and survived on BAR-type plants and weighed only 4.11 ± 0.02 mg.

In contrast to *M. brassicae* larvae*, P. rapae* larvae grew and survived equally well on both chemotypes. *P. rapae* larval biomass accumulation over time showed no significant difference between the chemotypes over the first 18 days (Figure 4.2B; Mann-Whitney *U* Test: $P > 0.05$ for each day of recording) and was significantly and positively correlated to leaf consumption after four days (Correlation: $r = 0.768$, $r^2 = 0.589$, $P < 0.01$). Leaf damage due to *P. rapae* feeding was not significantly different between the chemotypes (Figure 4.3B; Mann-Whitney *U* Test: $U = 673$, $Z = -1.217$, $P = 0.223$). After eight days larval mortality was 7.5% on NAS-type and 12.5% on BAR-type plants. On day 14, the first *P. rapae* larvae started pupating. After 16 days on BAR-type plants 45% had pupated and on NAS-type plants this was 47.5 %. At the end of the experiment, after 18 days, larval weights were still not significantly different on the chemotypes (on BAR-type: 161.9 ± 7.37 mg, on NAS-type: 175.24 ± 5.63 mg; Mann-Whitney *U* Test: $U = 238$, $Z = -1.131$, $P = 0.265$).

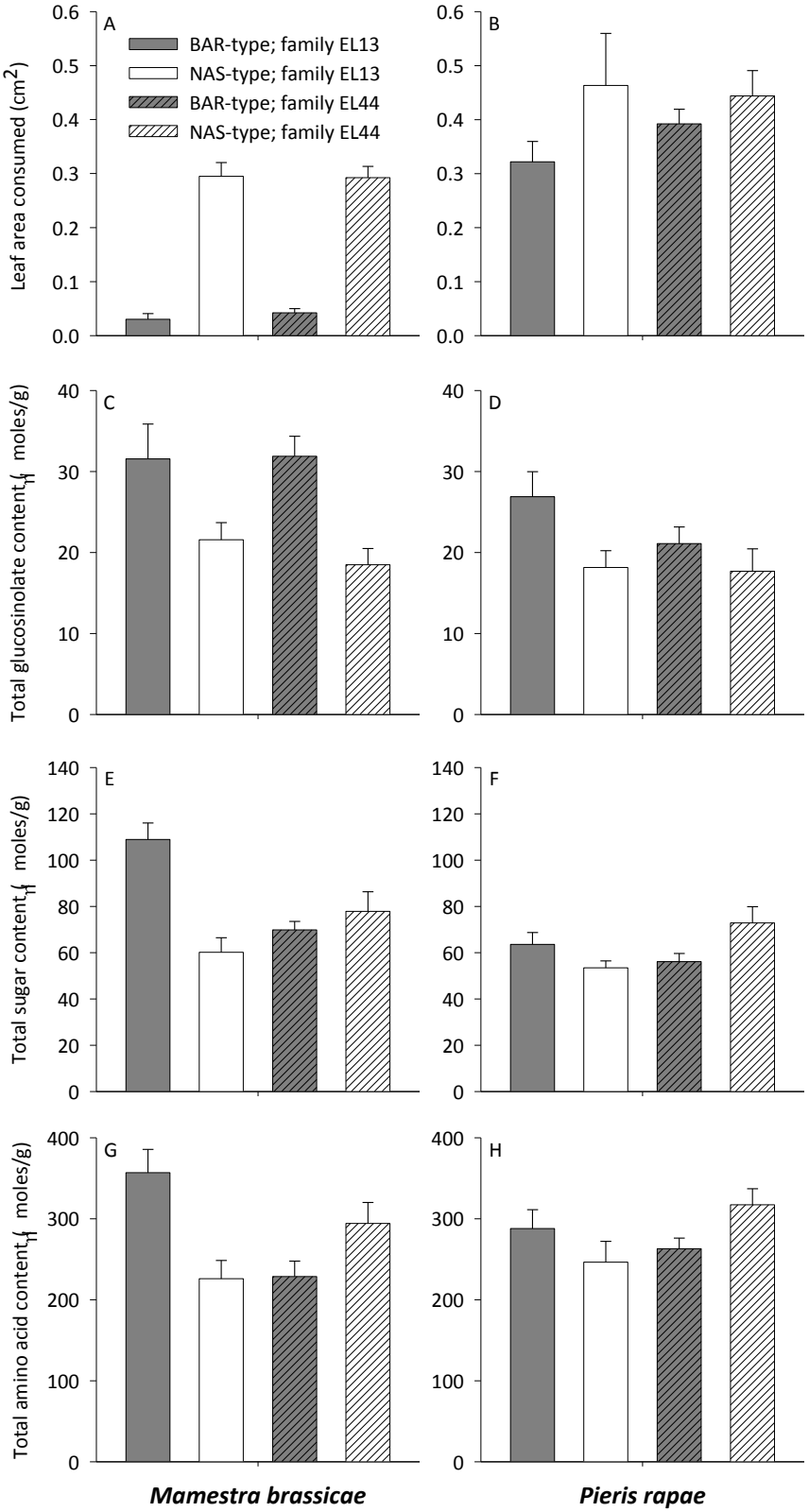

Figure 4.3 Leaf characteristics after 4 days of larval feeding of *Mamestra brassicae* (left graphs) or *Pieris* rapae (right graphs) on *Barbarea vulgaris* +/- SEM: (A & B) consumed leaf area (cm^2), (C & D) total glucosinolate content, (E & F) total sugar content, (G & H) total amino acid content. Results are depicted per chemotype (grey bars = BAR-type plants; white bars: NAS-type plants) and per half-sib family (no hatching = EL13, hatching = EL44).

Larval performance and total glucosinolate content

The leaves on which the larvae were feeding differed in glucosinolate composition (glucobarbarin versus gluconasturtiin) and in total glucosinolate content (Figure 4.3C & 4.3D). In both chemotypes, we detected glucobarbarin, gluconasturtiin, glucosibarin, glucobrassicin, 4-methoxyglucobrassicin and neoglucobrassicin. In both families, BAR-type leaves had on average 1.5 times higher total glucosinolate levels than NAS-type leaves (Figure 4.3C; ANOVA Type effect: $F_{1,143}$ = 22.831, P < 0.001). Because *M. brassicae* fed more on NAS plants, overall we found a negative - but weak - correlation of total glucosinolate content with the consumed leaf area (Correlation: $r(X,Y)$ = -0.26, r^2 = 0.065, P < 0.05). To analyze in more detail whether total glucosinolate level is determining larval consumption and performance, we also examined the correlation *within* each chemotype. Within chemotypes, the range of glucosinolate concentrations was substantial (factor 6-7), but there was no negative correlation between glucosinolate level and larval biomass (Figure 4.4; Correlation: within BAR, $r(X,Y)$ = 0.15, r^2 = 0.024, P = 0.932; within NAS, $r(X,Y)$ = 0.28, r^2 = 0.078, P = 0.090). Moreover, larvae on BAR-type leaves with similarly low levels of glucosinolates as NAS-types, still consumed and performed considerably worse (Fig 4.4 glucosinolate levels < 35 µmoles·g^{-1} dry mass). There was also no difference in total glucosinolate level between BAR-type leaves on which the larvae survived or died (with living larvae: 31.32 ± 2.95 µmoles·g^{-1}, with dead larvae: 32.81 ± 6.49 µmoles·g^{-1}; ANOVA: $F_{1,29}$ = 0.059, P > 0.05; Figure 4.4). As the performance of *P. rapae* larvae was similar among chemotypes and families, there was no correlation of larval biomass with total glucosinolate level.

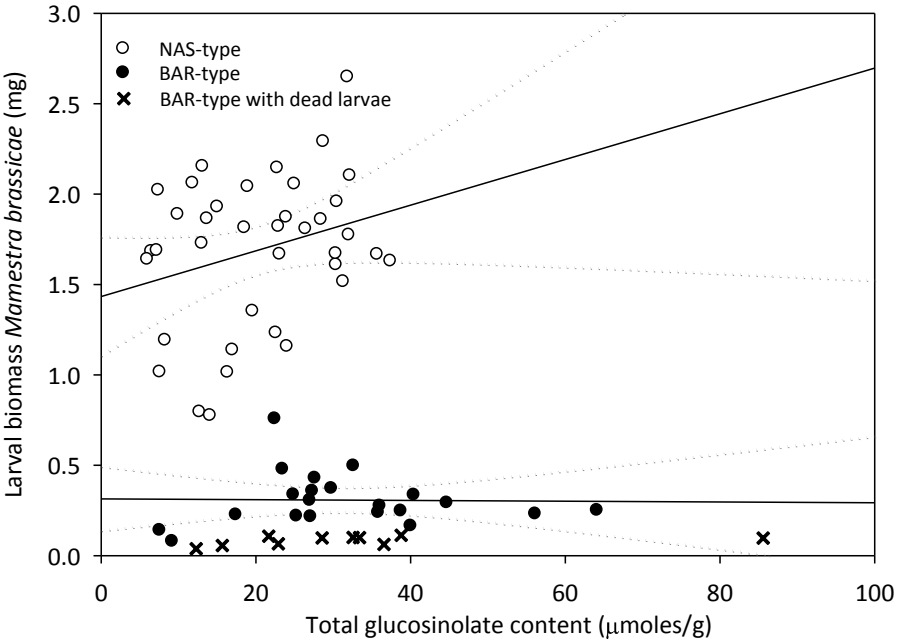

Figure 4.4 Correlation of total glucosinolate content and the larval biomass after 4 feeding days of Mamestra brassicae experiment on NAS-type (white dots), BAR-type (black dots) and dead larvae of on BAR-type (black crosses) Barbarea vulgaris plants with their correlations per chemotype (black line) and their 95% confidence intervals (dotted lines).

Larval performance and nutritional value

Total sugar content of both chemotypes was composed of five consistently detected sugars, which were glucose, sucrose, sorbitol, fructose and trehalose (from high to low average concentration). Total amino acid content of both chemotypes was composed of 11 consistently detected amino acids, which were threonine, isoleucine, arginine, serine, glutamate, aspartate, glutamine, asparagine, phenylalanine, tyrosine, and histidine (from high to low average concentration). Because the individual sugars and amino acids showed similar effects as the total levels (results not shown), we only present data on the total levels.

In contrast to the total glucosinolate level, sugar and amino acid content did not coherently differ between the chemotypes (Figure 4.3E-4.3H). Although in the *M. brassicae* experiment the total sugar content was overall higher in BAR-type plants, there was a significant interaction between chemotype and family (Figure 4.3E; ANOVA type effect: $F_{1, 75} = 12.014$, $P < 0.001$; type × family $F_{1, 75} = 10.252$, $P < 0.05$). Similar interactions between chemotype and family were shown for amino acid content of the plants in the *M. brassicae* experiment and for sugars and amino acid content of plants in the *P. rapae* experiment. In all chemotype × family interactions, the BAR-type of the EL13 half sib family had higher levels of primary metabolites than the NAS-type of that family (Figure 4.3E-3H). Remarkably, in the EL44 half-sib family the sugar and amino acid level differences of the chemotypes were negligible in plants during the *M. brassicae* experiment or even opposite to those of the EL13 during the *P. rapae* experiment. As sugar and amino acid content did not vary consistently with chemotype, sugars and amino acids cannot explain the differences in larval performance and preference between the chemotypes.

Discussion

NAS and BAR chemotypes of *B. vulgaris* differentially affected preference and performance of the generalist herbivore *M. brassicae* but not of the specialist *P. rapae*. Larvae of *M. brassicae* grew exponentially and had a 100% survival on NAS-type leaves, but hardly grew and had a high mortality when feeding on BAR-type plants. As a dose-dependent effect of total glucosinolate content on herbivores is well-known (Mauricio, 1998; Li et al., 2000; Agrawal and Kurashige, 2003; Mewis et al., 2006) the poor performance of *M. brassicae* could have been caused by the on average 1.5 times higher total glucosinolate content of BAR-type plants compared to NAS-type plants (Van Leur et al., 2006). However, we did not observe any dose-dependent effect *within* the chemotypes. Moreover, *M. brassicae* larvae on BAR-type plants with similar total glucosinolate levels as NAS-type plants still performed significantly worse. Hence, we can exclude total glucosinolate level as explanatory factor for the differences found between the chemotypes on larval biomass and leaf consumption.

In addition to total glucosinolate content the sugar and amino acid contents differed between chemotypes, but the pattern was inconsistent among half-sib families. The statistical interaction between chemotype and family found for sugar and amino acid levels was not found for herbivore performance and preference (Fig 4.3.). Therefore, total sugar content and total amino acid content could not explain the observed effects on *M. brassicae*.

In a different *B. vulgaris* polymorphism, described by Agerbirk et al. (2003a), there were P-type and G-type plants which differed in resistance to *Plutella xylostella*. Other than our BAR/NAS polymorphism, the P-type and G-types also differed in trichome density, did not co-occur in natural populations, were hard to cross, and neither of the Danish types had a high content of gluconasturtiin in the leaves (N. Agerbirk, personal communication).The resistance of the G-type to *P. xylostella* was found to be due to a difference in saponin content (Agerbirk et al., 2003b). Based on these findings, we compared saponin levels between BAR and NAS-type plants using LC-TOF-MS. This analysis revealed that the levels of the saponin described by Agerbirk et al. (2003b) and a saponin described by Shinoda et al. (2002) did not differ between the NAS and BAR chemotypes (Chapter 3). Therefore we can exclude these saponins as explanatory factor for the poor performance of *M. brassicae* on BAR-type plants.

Consequently, the observed effects are likely to be caused by the difference in glucosinolate structure and their break-down products. BAR-type plants were more toxic and deterrent to *M. brassicae* than the NAS-type plants. This might suggest that *M. brassicae* can deal effectively with gluconasturtiin and isothiocyanates, but not with glucobarbarin and resulting oxazolidine-2-thiones. The latter compounds have received hardly any attention in chemical-ecological literature. Generalist herbivores possess broad-spectrum detoxification enzymes such as P450 enzymes and mixed-function oxidases (MFO) enabling them to deal with a wide range of allelochemicals, including glucosinolates (Schoonhoven et al., 1998b; Li et al., 2000; Mainguet et al., 2000). Which detoxification mechanism is present in *M. brassicae* and why it can handle the presumably more toxic isothiocyanates, but not the oxazolidine thiones is unknown. The non-different performance of *P. rapae* on the chemotypes may indicate that the NSP enzymes are equally effective in redirecting the hydrolysis pathways of both chemotypes towards the generally less toxic nitriles (Wittstock et al., 2004). Further identification of the bioactive compounds could be acquired using bioassay-guided fractionation.

Neither herbivore species significantly preferred one of the chemotypes as host plant for oviposition. Even though isothiocyanates are known to elicit anemotaxis in herbivores at extremely low concentrations (Finch and Skinner, 1982), the concentrations of volatile cues emitted by our undamaged plants may have been below the detection limit (Finch et al., 1978). Upon damage or induction by herbivores volatile levels may rise and affect oviposition preference (Rothschild and Schoonhoven, 1977; Anderson and Alborn, 1999; Bruinsma et al., 2007). Next to chemical suitability, the surface on which to oviposit can be an important factor (Renwick and Chew, 1994; Gotthard et al., 2004). In our experiment, however, leaf surface structure was unlikely to affect oviposition preference between the *B. vulgaris* chemotypes because the chemotypes did not have visible differences in leaf surface (e.g. trichomes) in contrast to the completely different *B. vulgaris* polymorphism reported by Agerbirk (2003a).

As *M. brassicae* larvae performed extremely poorly on BAR-type plants, the lack of oviposition preference of *M. brassicae* adults seems to be non-adaptive. The discrepancy between larval performance and oviposition preference on *B. vulgaris* is also shown for the Diamondback moth (*Plutella xylostella)* (Serizawa et al., 2001; Badenes-Perez et al., 2006), and may occur when insects or plants are new to an area and there has not been enough

time for evolutionary adaptation (Agosta, 2006). Whether this is the case for *M. brassicae* and the *B. vulgaris* chemotypes is unknown.

Co-evolutionary theory suggests that the variation of plant defence compounds is maintained by sequential adaptations of specialist herbivores and plants (Agrawal et al., 1999; Cornell and Hawkins, 2003). The good performance of *P. rapae* on both chemotypes suggests that both chemotypes are not, or not anymore, effectively defended against this specialist. On the contrary BAR-type plants are effectively defended against the generalist *M. brassicae.* Although we have no experimental evidence indicating which chemotype is the evolutionary newest form, the effective defence of BAR-type plants against *M. brassicae* suggests an evolutionary adaptive step in which BAR-type had evolved from the NAS-type. This matches with the biosynthetic origin of these glucosinolates. Gluconasturtiin is presumably the precursor which is hydroxylated to produce glucobarbarin. Moreover, gluconasturtiin occurs in 5 times more genera than glucobarbarin (Fahey et al., 2001). This does not preclude, however, that the NAS-types found locally in the Netherlands are due to a loss-of-function mutation from the BAR-type. In natural populations of *B. vulgaris* there are only 0-22% NAS-type plants (Van Leur et al., 2006). This results in a potentially limited genetic basis for NAS-type plants compared to the BAR-type plants, which could contribute to linkage disequilibrium between the BAR/NAS locus and other loci (for instance closely linked loci). However the natural population that we selected to obtain seed batches for these experiments had a phenotype frequency close to a Hardy-Weinberg equilibrium (22% potential NAS-type fathers observed instead of 25% expected). We therefore estimate the chance of linkage disequilibrium effects in our experiments relatively small. Additionally there are no *a priori* indications of closely linked loci which affect herbivore performance

For the plant the benefits of increasing its defence against *M. brassicae* will be larger than increasing defence against *P. rapae*. Being gregarious and larger, *M. brassicae* larvae are more harmful to the chosen plant individual (many larvae feeding from 1 plant) and will stronger reduce plant fitness than the solitary *P. rapae* (only 1 or few larvae feeding on 1 plant). Therefore, irrespective of the degree of specialization of the herbivores, it will be more important for *B. vulgaris* to be defended against *M. brassicae* than against *P. rapae.*

Based on our results we would expect BAR-type plants in the field to suffer less from herbivory than NAS-type plants. Besides being the chemotype that is most severely damaged by *M. brassicae* larvae, the NAS chemotype is also the recessive genotype. Therefore, when herbivorous insects are the only selective force, we expect that natural selection will drive natural populations towards 100% BAR-types. European populations of *B. vulgaris* indeed are mainly dominated by BAR-type plants, but a minority of populations has still up to 22% NAS-type plants. This indicates that in these populations there may be other factors playing a role in maintaining this chemical polymorphism. Therefore, other factors that may differ between the two types and which determine plant fitness, e.g., belowground herbivory, higher trophic level interactions and inter- and intraspecific competition, need to be included in future studies.

Acknowledgements

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CHAPTER 5

Reciprocal interactions between the Cabbage root fly (*Delia radicum*) and two glucosinolate phenotypes of *Barbarea vulgaris*

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Submitted

Abstract

The cabbage root fly (*Delia radicum* L.*,* Diptera: Anthomyiidae) has a life cycle which is spatially separated. Adults live and oviposit aboveground whereas larvae feed and pupate belowground. Oviposition choice is affected by shoot glucosinolates. However, little is known about belowground plant defence against *D. radicum*. Here, we use two naturally occurring heritable chemotypes of *Barbarea vulgaris* R. Br., which have different glucosinolate patterns, to investigate the effect of glucosinolates on the preference and performance of *D. radicum*.

The performance was studied by applying ten neonate larvae·plant^{-1} and measuring pupal biomass after 18 days. There was no difference in retrieval, but pupae had a higher biomass when having developed on BAR-type plants. Choice experiments showed no significant oviposition preference of *D. radicum* for either chemotype. However, there was a trend towards preference for the BAR-type. In a separate experiment we compared the physiological response of both chemotypes to *D. radicum*. Infestation reduced root and shoot biomass, root sugar and amino acid levels, as well as shoot sugar levels. Except for shoot sugar levels, these responses did not differ between the two chemotypes. Shoot or root glucosinolate patterns did not change upon infestation. As glucosinolate patterns were the only consistent difference between the chemotypes, it is likely this difference caused the reduced biomass of *D. radicum* pupae on NAS-type plants.

In a garden experiment, plants were severely damaged by root flies, but we found no differences in the percentage of fallen over flower stalks between the chemotypes. We found more pupae in the soil near BAR-type plants, but overall this was not statistically significant. The results of the preference and performance experiments in the greenhouse, combined with the retrieval data in our garden experiment, suggest that BAR-type plants overall are more suitable hosts than NAS-type plants.

Keywords

Root fly, crucifer, plant defence, glucosinolates, glucobarbarin, gluconasturtiin, isothiocyanate, polymorphism, sugars, amino acids.

Introduction

Plants have to deal with aboveground and belowground herbivores. In most cases, herbivores interacting with roots and shoots belong to different species, but in some cases they are different life stages of a single species. The Cabbage root fly (*Delia radicum* L.*,* Diptera: Anthomyiidae) has a life cycle in such spatially separated domains. *D. radicum* is a severe pest of crucifer plants in natural and in agricultural systems (Finch and Ackley, 1977; Finch, 1993). The adult flies live aboveground and gravid females select suitable oviposition sites based on aboveground visual and olfactory cues (Nottingham, 1988; Roessingh and Städler, 1990; Marazzi and Städler, 2004). Eggs are deposited near the root-stem interface. After hatching the larvae crawl into the soil and start feeding from the upper roots of the host plant. Pupae develop in the soil around the roots, and after emergence, the adult flies return aboveground (Block et al., 1987).

Studies on *D. radicum* host-plant interactions have been focussing mainly on the oviposition behaviour of female flies. These studies showed that egg deposition is tightly linked with chemical cues indicating host plant suitability. Important chemical cues are two so called CIF factors identified on the leaf surface of *Brassica oleracea* and in roots of *Brassica napus* (Baur et al., 1996; Hurter et al., 1999; de Jong et al., 2000). Next to these CIF factors, shoot glucosinolates are shown to play a role in oviposition host selection (Hardman and Ellis, 1978; Roessingh and Städler, 1990; Städler and Schoni, 1990; Roessingh et al., 1992; Hopkins et al., 1997). Glucosinolates are a group of crucifer-specific plant defence compounds, which hydrolyse upon cell disruption and form various breakdown products (Louda and Mole, 1991; Mithen, 2001a; Halkier and Gershenzon, 2006). Besides the reaction conditions, such as pH, the exact chemical structure of the glucosinolate determines which breakdown product is formed (Chew, 1988; Fahey et al., 2001; Wittstock et al., 2003). Electrophysiological and behavioural studies have shown various glucosinolates and their breakdown products to be detected by *D. radicum* flies. Moreover, the oviposition response depends on the content of individual glucosinolates or breakdown products, rather than on total glucosinolate levels (Nair et al., 1976; Ellis et al., 1980; Roessingh et al., 1992; Städler et al., 2002).

Even though there are host-suitability studies using different species of Brassicaceae (Finch and Ackley, 1977), it has never been explicitly studied how different root glucosinolates affect the larval performance of *D. radicum.* In the present study, we link oviposition preference to larval performance of *D. radicum* on two well-defined glucosinolate chemotypes of *Barbarea vulgaris*. The glucosinolate polymorphism in *B. vulgaris* was found in natural populations in The Netherlands and consists of two heritable chemotypes van Leur et al. (2006). The most common and genetically dominant glucosinolate profile is dominated (94% of shoot glucosinolates) by glucobarbarin (BAR-type), whereas in the recessive phenotype gluconasturtiin (NAS-type) is most prominent (82%). Because of the different glucosinolates, BAR-type and NAS-type plants are, upon damage, expected to form different hydrolysis products. Gluconasturtiin most likely forms the volatile 2-phenylethyl-isothiocyanate (Musk et al., 1995; Barillari et al., 2001; Canistro et al., 2004b). Glucobarbarin on the other hand produces (*S*)-2-hydroxy-2-phenylethyl-isothiocyanate, but due to the 2-hydroxylation the isothiocyanate is unstable and spontaneously cyclizes to a less volatile 5-phenyloxazolidine-2-thione (Kjaer and Gmelin, 1957). Next to the aforementioned qualitative difference, there is a quantitative difference: seeds, flowers and rosette leafs of

BAR-type plants have a higher total level of glucosinolates (Van Leur et al., 2006). Although the differences between the chemotypes are less distinct in roots, the chemotype is consistent in all tissues (Van Leur et al., 2006). *B. vulgaris*, probably the most common BAR-type, was found to be a suitable host for *D. radicum* larvae (Finch and Ackley, 1977; Städler et al., 2002). However, due to the differences in glucosinolate profiles we hypothesize that roots, as well as shoots of the *B. vulgaris* chemotypes differentially influence *D. radicum.* The *B. vulgaris* glucosinolate polymorphism is an ideal system to test the effect of different glucosinolates on *D. radicum* preference and performance. Because we compared performance on NAS and BAR plants within a half sib family, we were able to reduce the variation in genetic background (Strauss et al., 2002). In natural populations, the outcome of herbivore-plant interactions may differ from in the lab, because the interactions take place in a framework of a complex and dynamically changing environment. Therefore, we studied the interactions between *D. radicum* and the two *B. vulgaris* chemotypes also under semi-field conditions in an experimental garden.

Plants are not static victims of herbivores but are known to respond locally and systemically by increasing their defence levels (Bezemer and van Dam, 2005). In response to *D. radicum* infestation, cultivated *Brassica* species showed changes in root and shoot biomass, glucosinolate content (Van Dam and Raaijmakers, 2006) and primary metabolites levels (Hopkins et al., 1999). Therefore, root and shoot biomass and glucosinolate, soluble sugar and amino acid levels of the *B. vulgaris* chemotypes were analyzed under controlled conditions and upon infestation with *D. radicum*. The local responses in the root as well as the systemic responses in the shoot were measured.

Materials and Methods

Plant and insect rearing

B. vulgaris seeds were collected from ten individual BAR-type and their nearest neighbour NAS-type maternal plants, which were freely cross-pollinated in a natural population of *B. vulgaris*. The population was located in Elderveld, The Netherlands (51.95 °N; 5.87 °E) and consisted of 22% NAS-type plants (Van Leur et al., 2006). We selected offspring of maternal NAS-type plants “EL44” (68% BAR-type offspring) and “EL13” (62% BAR-type offspring). Plants were grown in a glasshouse, at 21 °C (day) and 16 °C (night), with 60% relative humidity and natural daylight supplemented with sodium lamps to maintain the minimum PAR at 225 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a photoperiod of 16:8 (L:D). One week after germination on glass beads, the seedlings were transplanted to a mixture of peat soil (Potgrond 4, Lentse Potgrond BV., Lent, The Netherlands) and 20% sand. Two-weeks-old seedlings were transplanted to 1.1L pots, watered and fertilized regularly with half strength Hoagland’s nutrient solution with a doubled KH_2PO_4 content.

Delia radicum (root herbivore) larvae were obtained from cultures maintained on *Brassica napus* roots at the Netherlands Institute of Ecology, Heteren. Adult root flies were obtained from pupae cultured at the Laboratory of Ecobiology of Insect Parasitoids at Rennes University in France.

Effect of plant chemotype on oviposition preference

Oviposition preference of *D. radicum* was tested on six-months-old BAR-type and NAS-type rosette plants from half sib family EL44. The shoots of the plants were clipped one month prior to use, to ensure abundant fresh leaf material. Under greenhouse conditions (see above) a stock colony of adult flies was kept in a mesh cage (40 × 45 × 65 cm^3). Water was supplied on water-soaked cotton wool. Nutrition consisted of a mixture of 1/3 milk powder, 1/3 sugar and 1/3 yeast flakes. Males and females were held together to mate. Oviposition preference was assessed by introducing ten randomly chosen adult flies from the stock colony into one of the oviposition mesh cages (40 × 45 × 65 cm^3) each containing one BAR-type and one NAS-type plant. Only one fresh full-grown leaf per plant was exposed to the flies, the rest of the plant was covered with air and water permeable non-woven polypropylene (17gr/m^2, Hanovlies ®, HANOTEX BV., Joure, the Netherlands). The petiole of the uncovered leaf was surrounded by a felt collar (ca 6 cm diameter) which was watered regularly to fulfil the preference of the flies for an oviposition substrate of 60% humidity in which it can grub. One, two and three days after the flies had been introduced in the cages, the eggs on the felt collar of each plant were counted and removed. After three days, both the plants and the flies were removed from the test cages, and new plants and flies were introduced. After three test series, we obtained data on 30 replicate oviposition choice experiments.

Effect of plant chemotype on root fly performance

To asses *Delia radicum* performance on the two *B. vulgaris* chemotypes, the plants of the oviposition experiment were each infested with ten neonate *Delia radicum* neonate larvae as described for the first experiment. After 18 days pupae were collected by washing-out the roots and surrounding soil. Searching time was limited to 15 min.·$plant^{-1}$. The retrieved pupae were frozen at -20˚C and subsequently lyophilized and weighed to determine the dry mass (DW).

Effect of root fly on plant biomass

Five late L1 to early L2 *D. radicum* larvae were added to 32 BAR-type and 32 NAS-type *B. vulgaris* rosette plants, belonging to half sib family EL44 (50%) or EL13 (50%). The larvae were transferred to the plants by placing the larvae with a brush on a wedge of heavy weight filter paper (300 g/m) that was saturated with water. The tip of the wedge was inserted into the soil next to the plant at ca 1 cm from the root-stem interface (Finch and Ackley, 1977; Van Dam et al., 2005). Three hours later, all larvae had disappeared into the soil and the filter paper was removed from the pots. After five and twelve days, eight plants per infested treatment group were harvested and the roots were carefully washed to remove the soil. Larvae and pupae were collected from the washed-out soil and from the roots. Searching time was limited to 20 min.·$plant^{-1}$. The roots and shoots were oven-dried at 70 °C and, subsequently, weighed on a microbalance to determine total root and total shoot dry mass.

Plant chemical composition

To determine the plant's response to *D. radicum* infestation, an additional set of plants was treated as described in the biomass experiment. This set consisted of five infested and five control plants per chemotype per half sib family. Control plants received a wedge of filter paper without larvae. All plants of this additional set were harvested in between the two other harvests at day 7. These plants were checked for root fly damage, evidenced by brown/orange wounds and the presence of galleries. Roots and shoots were sampled. As most damage was detected at the main root close to root-stem interface, this part of the root (2 cm length) with the first 2 cm of the attached smaller roots was collected both in the infested and control group. The complete shoot was sampled. All samples were frozen at -20˚C immediately after harvesting and subsequently lyophilized and ground to a fine powder in a ball mill (Retsch, type MM301, Retsch GmbH & Co., Haan, Germany) and stored dry and in the dark until extraction.

Root fly infestation in an experimental garden

In April 2005, a semi-field condition experiment was started in an experimental garden in Heteren, the Netherlands. Six hundred, two-months-old plants of which the maternal plants originated from natural populations in Heteren, Elderveld and Oosterbeek (van Leur et al. (2006) were selected and planted in the experimental garden. Positioning of the plants in the field was randomized for population origin, but the chemotypes were placed in an alternated design. All plants were separated by 0.5 meter bare soil and placed in 25 rows, with 24 plants per row resulting in one big plot of 12 × 13 meter. In April, July and October 2005 and April, June, August and October 2006 six plants per population origin per chemotype were harvested (36 plants total per harvest). Shoots were removed and roots were dug up so that we extracted a fixed volume (20 × 20 × 20 cm^3) surrounding the roots, resulting in app. 7 kg soil/root mass. This soil/root sample was stored in plastic bags at 4˚C for a maximum of three days. The roots were washed and soil was filtered in a mesh sieve to retrieve all *D. radicum* pupae. A subset of the pupae was reared and the derived flies were confirmed to be *D. radicum* by Y. Jongema of the Laboratory of Entomology (Wageningen University, The Netherlands).

As a measure of plant damage in the field, the percentage of flower stalks which had fallen over per plant was scored. This was done weekly of a subset of 68-90 plants (5 rows) per week from 06-21-2006 till 07-20-2006. In this period all plants were measured only once.

Chemical analysis

For quantification of glucosinolates, soluble sugars and amino acids, one global extraction was used. In a 2 ml Eppendorf tube 50.0 mg of lyophilized, finely ground plant material was dissolved in 1.0 ml 70% MeOH in water (v/v), vortexed and immediately boiled for five min. to kill remaining myrosinase activity. Tubes were placed in an ultrasonic bath for 15 min. and centrifuged (10 min. 10000 r.p.m). The extraction was repeated for the pellet omitting the boiling step. Both supernatants were combined per sample in a new 2 ml Eppendorf tube and supplemented individually with 70% MeOH to attain the average mass (n = 3) of a 2 ml

Eppendorf tube containing 2.0 ml 70% MeOH. This "stock" extract was stored at -20 °C until further analysis.

Half (1.0 ml) of the stock extract was used for glucosinolate analysis and applied to a DEAE-Sephadex A 25 column (EC, 1990), desulphated with arylsulphatase (Sigma, St. Louis, IL, USA) and separated on a reversed phase C-18 column on HPLC with a CH_3CN–H_2O gradient as described in van Dam et al. (2004). Glucosinolate analysis was performed with a PDA detector (200–350 nm) with 229 nm as the integration wavelength. Desulfoglucosinolate peaks were identified by comparison of HPLC retention times and UV spectra with authentic standards isolated from *B. vulgaris* as previously described (Agerbirk et al., 2001b), as well as standards kindly provided by M. Reichelt, MPI Chemical Ecology, and a certified rape seed standard (Community Bureau of Reference, Brussels, code BCR-367R). For glucobarbarin, we used the same response factor as for gluconasturtiin. To calculate glucosinolate concentrations in the plant tissue, the obtained values were multiplied by two and divided by dry mass.

To analyze soluble sugar content, a 10 µl aliquot of the stock extract was diluted with 990 µl MilliQ water. Soluble sugars were analyzed by injecting 5 µl of the diluted extract on Dionex HPLC system, equipped with a Carbopac PA1 column (2 × 250 mm) and a Carbopac PA1 guard column (2 × 50 mm, Dionex Corp. Sunnyvale CA, USA). An isocratic gradient mixture of 10% 1 M NaOH and 90% MilliQ water was used to separate the sugars at a flow rate of 0.25 ml/min. Column temperature was kept at 20 °C. A "10 ppm" reference solution containing 54.9 µM sorbitol and manitol, 29.21 µM trehalose, sucrose and melbiose, and 55.51 µM glucose and fructose, was diluted to obtain 7.5 ppm, 5 ppm and 2.5 ppm calibration standards to obtain a reference curve. After every 10 samples an additional standard was injected to check for deviations of retention times and the calibration curve. To calculate the molar concentration of sugars in the plant tissue, the concentration values were multiplied by 200 and divided by dry mass.

Amino acids were analyzed on a Dionex HPLC system by integrated pulsed amperometric detection. An aliquot of 20 µl of the stock extract was diluted with 980 µl MilliQ. Of this diluted extract 25 µl was injected and amino acids were separated with a ternary gradient (see DIONEX application update 152, Method 1, standard AAA gradient; condition 60/2 in Hanko and Rohrer, 2004) on a 2 × 250 mm AminoPac© PA10 column with a 2 × 50 mm AminoPac© PA10 Guard column (Dionex, Sunnyvale,CA, USA). Eluents, flow rates, waveform and working electrode conditions were all as specified under Method 1 in Dionex application update 152 and in Hanko and Rohrer (2004). The Sigma AA-S-18 amino acid standard (Sigma, St Louis, MO, USA) containing 17 amino acids was supplemented with asparagine, glutamine and tryptophan (2.5 µmoles/ml each) to obtain a reference sample containing the 20 most common amino acids. This reference solution was diluted to obtain calibration standard ranging from 1 – 8 µM for each amino acid, except for cysteine, which had a range of 0.5 – 4 µM. After every 10 samples an additional standard was injected to check for deviations of retention times and the calibration curve. To calculate the molar concentration of the amino acids in the plant tissue, the concentration values were multiplied by 200 and divided by dry mass.

To determine the chemotype of each plant, glucosinolates were extracted from the first full grown leaf and analysed on HPLC as described above. When the peak area of glucobarbarin divided by the peak area of gluconasturtiin was > 10, the plant was considered a BAR-type, when this ratio was < 0.1 it was considered a NAS-type.

Statistical analysis

Statistical analyses were performed with STATISTICA (data analysis software system), version 8.0 (StatSoft, Inc., Tusla, OK, USA). Normality and homogeneity of variance were checked by inspection of the residuals. Oviposition preference was tested with a Sign test and the numbers of eggs were compared between the chemotypes with a T-test for dependent samples. The dry masses of the retrieved pupae were averaged per plant and tested for chemotype effect using a T-test for independent samples. The field data for number of retrieved pupae and percentage of infested flower stalks did not fit a normal distribution and were therefore tested with a non-parametrical Kruskal Wallis tests and a Mann-Whitney U test respectively.

The overall effects of *D. radicum* feeding on root and shoot biomass, as well as the number of retrieved larvae and pupae were analysed using factorial MANOVAs, with harvest time, chemotype and half-sib family as main factors. MANOVAs showing significant effects were followed by univariate ANOVAs to analyse which of the dependent variables were affected by *D. radicum* feeding. Prior to analysis of variance (ANOVA) total glucosinolate, sugar and amino acid content data were square root transformed to meet the assumptions of ANOVA.

Results

Effect of plant chemotype on oviposition preference

D. radicum female flies oviposited in 20 out of 30 replicate choice experiments. In 13 of the replicates most eggs were deposited on BAR-type plants, and in only 7 replicates NAS-type plants were preferred. Per replicate a total of 22.6 (± 3.81 SE) eggs were deposited, whereby BAR-type plants received on average 1.77 times the number of eggs on NAS-type plants (Figure 5.1). Although there was a trend towards a preference for BAR-type plants, neither the number of eggs·plant^{-1} nor the absolute preference was significantly different between the chemotypes (T-test for Dependent Samples on # eggs n = 20, P = 0.20; Sign test on preference Z = 0.72, P = 0.472).

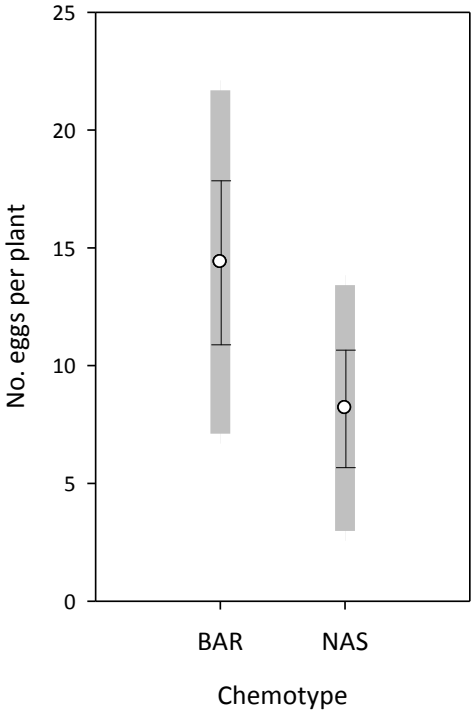

Figure 5.1 Oviposition of *Delia radicum* on BAR-type and NAS-type *Barbarea vulgaris* plants. Black error bars indicate standard errors; the grey area indicates 95% confidence intervals.

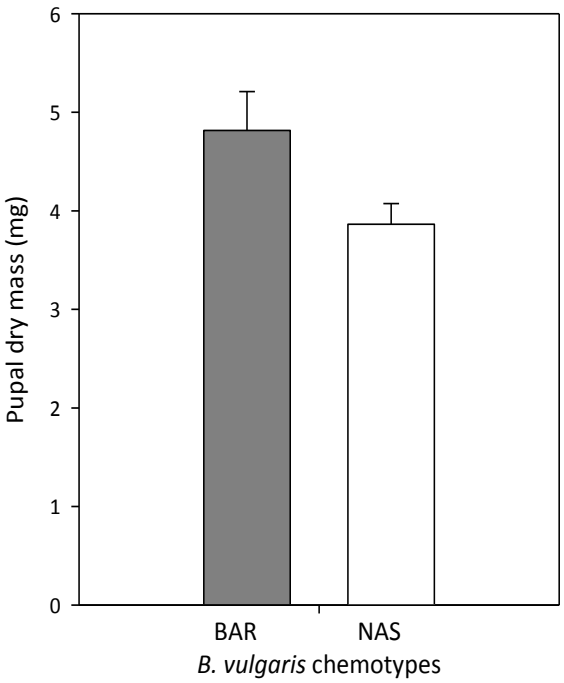

Figure 5.2 Dry mass of *Delia radicum* pupae on BAR-type and NAS-type *Barbarea vulgaris* plants. Error bars indicate standard errors.

Effect of plant chemotype on root fly performance

Despite a similar low retrieval for both chemotypes (overall 0.77 ± 0.14 out of 10 larvae), pupal biomass differed between the two *B. vulgaris* chemotypes. Larvae developed on BAR-type plants produced pupae with a significantly higher dry mass than those on NAS-type plants (Figure 5.2; ANOVA type effect: $F_{1,23} = 4.70$, $P < 0.05$).

Effect of root fly on plant biomass

Roots of every plant harvested at days 5 and 12 showed galleries and brown-orange colouring. Therefore, infestation with larvae of *D. radicum* was considered successful for all plants. On 94% of the plants, one or more *D. radicum* individuals were retrieved. The average retrieval per plant was 56%. Compared to the harvest after five days, the harvest after twelve days yielded significantly less larvae per plant (1.19 ± 0.18 at day 5 vs 0.44 ± 0.12 at day 12) but more pupae (1.63 ± 0.26 at day 5 vs 2.41 ± 0.25 at day 12) (Table 5.1). Next to harvest time, larval retrieval was affected by the half sib family to which the plants belonged to. More larvae were retrieved from EL13 plants than from EL 44 plants. Due to a relatively high retrieval on EL13 BAR-type plants after twelve days (0.88 ± 0.30), there was an interaction between family, type and time for larval retrieval (Table 5.1). This statistical three-way interaction was not found for pupal retrieval. Larval or pupal retrieval did not differ between the chemotypes. Therefore, we assume a similar infestation for each chemotype which enables us to compare biomass, glucosinolates and primary metabolites between both chemotypes within each half-sib family.

Table 5.1 F values and significance levels (*$P < 0.05$, ** $P < 0.001$) of an ANOVA on retrieval of larvae and pupae after five and twelve days (Week) on two half sib families (Family) with each two chemotypes (Type) of *Barbarea vulgaris.*

	Type	Family	Week	Type*Family	Family*Week	Type*Week	Type*Family*Week
retrieval larvae	0.42	10.29*	14.82 **	1.65	1.65	0.41	6.59*
retrieval pupae	0.87	0.59	4.51*	0.58	0.35	0.87	0.35

Twelve days of infestation with *D. radicum* caused a substantial reduction of plant dry mass compared to five days of infestation. The reduction in root dry mass was 57% (Figure 5.3a; ANOVA time effect root DW: $F_{1,56} = 92{,}59$, $P < 0.001$) and in shoot dry mass 55% (Figure 5.3b; ANOVA time effect shoot DW: $F_{1,56} = 520{,}38$, $P < 0.001$). The dry mass of shoots and roots were significantly different between half-sib families (ANOVA family effect: root $F_{1,56} = 4.49$, $P < 0.05$; shoot $F_{1,56} = 7.76$, $P < 0.05$), but there were no interactions between half sib family and harvest time. There was no overall difference in biomass reduction between the chemotypes (ANOVA type effect: root $F_{1,56} = 0.59$, $P = 0.446$, shoot $F_{1,56} = 1.30$, $P = 0.259$). Due to the relatively large reduction in shoot biomass of BAR type plants from family EL44, a three-way interaction for family × type × time was recorded for the shoot but not for the roots (ANOVA family × type × time effect: root $F_{1,56} = 1.40$, $P = 0.242$, shoot $F_{1,56} = 8.73$, $P < 0.05$).

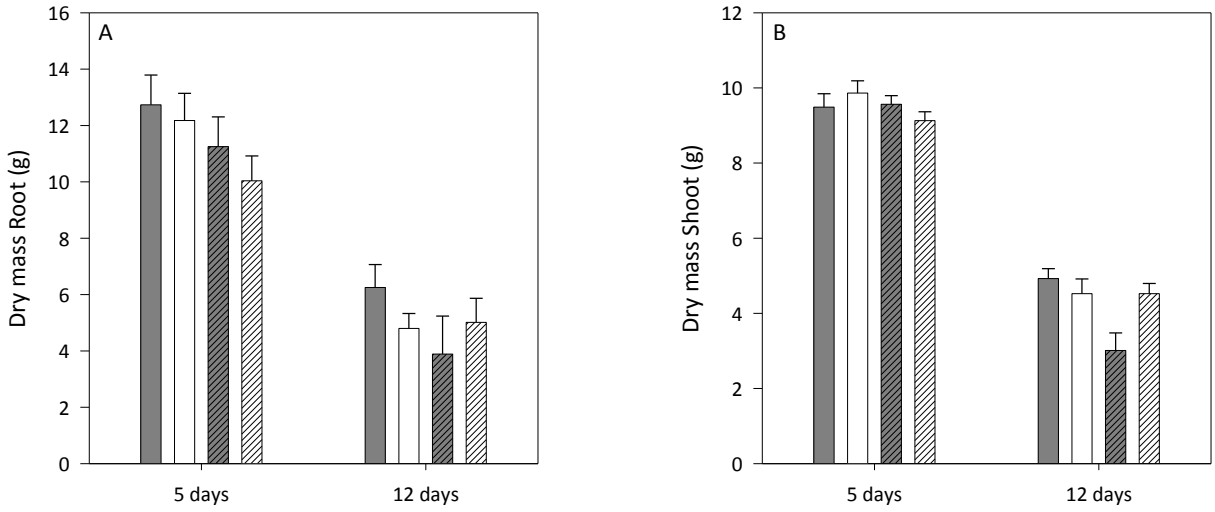

Figure 5.3 Root and shoot dry mass of *Barbarea vulgaris* BAR-type (grey bars) and NAS-type (white bars) plants from two half sib families (EL 13 not hatched, and EL 44 hatched bars) after 5 and 12 days of infestation with *Delia radicum* larvae. Error bars indicate standard errors.

Effect of root fly on plant chemical composition

Irrespective of *D. radicum* infestation, the chemotypes showed significantly different glucosinolate patterns (Figure 5.4a). BAR-type plants contained more glucobarbarin and less gluconasturtiin than NAS-type plants. The total levels of glucosinolates were higher in the roots of BAR-type plants. Shoots had approximately seven times lower total glucosinolate levels than the roots and did not show a significant difference in total glucosinolate levels. Upon *D. radicum* infestation, the total glucosinolate levels changed neither locally in the root (Figure 5.4a) nor systemically in the shoot (Figure 5.4b; Table 5.2). The same figures and table show that in both tissues the families had significantly different total glucosinolate levels; both in roots and shoots plants of EL13 had higher glucosinolate levels (Figure 5.4a and b, Table 5.2). Figures 5.4a and 5.4b also show that the glucobarbarin and gluconasturtiin levels are consistent for both half sib families and did not change upon infestation with *D. radicum*.

Table 5.2 F values and significance levels (*$P < 0.05$, ** $P < 0.001$) of an ANOVA on the total root and shoot levels of plant metabolites compared between infested and uninfested plants (Treatm.) of two half sib families (Family) with each two chemotypes (Type) of *Barbarea vulgaris.*

	Type	**Family**	**Treatm.**	**Type*Family**	**Family*Treatm.**	**Type*Treatm.**	**Type*Family*Treatm.**
Root							
Glucosinolates	6.06*	17.57**	0.32	0.21	1.38	0.36	0.25
Amino Acids	1.49	3.41	20.44**	0.93	0.14	0.31	0.76
Sugars	0.70	0.36	16.34**	0.58	0.55	1.70	0.34
Shoot							
Glucosinolates	0.16	8.56*	1.43	0.37	0.04	0.33	0.07
Amino Acids	0.02	7.18*	0.60	0.01	3.37	0.68	0.11
Sugars	0.01	1.53	9.69*	1.18	0.49	4.89*	3.55

Seven different soluble sugars (from high to low concentrations, averaged over roots and shoots: glucose, sucrose, fructose, sorbitol, manitol, threhalose and melbiose) and 13 soluble amino acids (from high to low concentration: isoleucine, tyrosine, proline, histidine, valine, arginine, asparigine, leucine, aspartate, glycine, cystine, lysine, alanine) were detected in *B. vulgaris* extracts. Seven other amino acids (phenylalanine, glutamine, threonine, tryptophan, glutamate, serine, and methionine) were detected occasionally and in very low amounts and were, therefore, not included in the analyses. The patterns of the individual amino acids and sugars followed those of the total levels, so that only the latter are presented. Upon infestation with *D. radicum* there was a significant local decline of the total levels of amino acids and sugars in the root (Figure 5.4c & 5.4e). For amino acids there was no systemic response (Figure 5.4d), but for sugars the shoot levels significantly decreased upon infestation (Figure 5.4f). However, there was a significant type × treatment interaction for shoot sugar content (Figure 5.4f, Table 5.2) which was due to a stronger reduction in NAS-type plants than in BAR-type plants. A family effect was present only for shoot amino acids, mainly because undamaged EL44 plants had higher levels than plants of EL 13 (Figure 5.4c and 5.4d). In contrast to the total glucosinolate level, sugar and amino acid content did not coherently differ between the chemotypes.

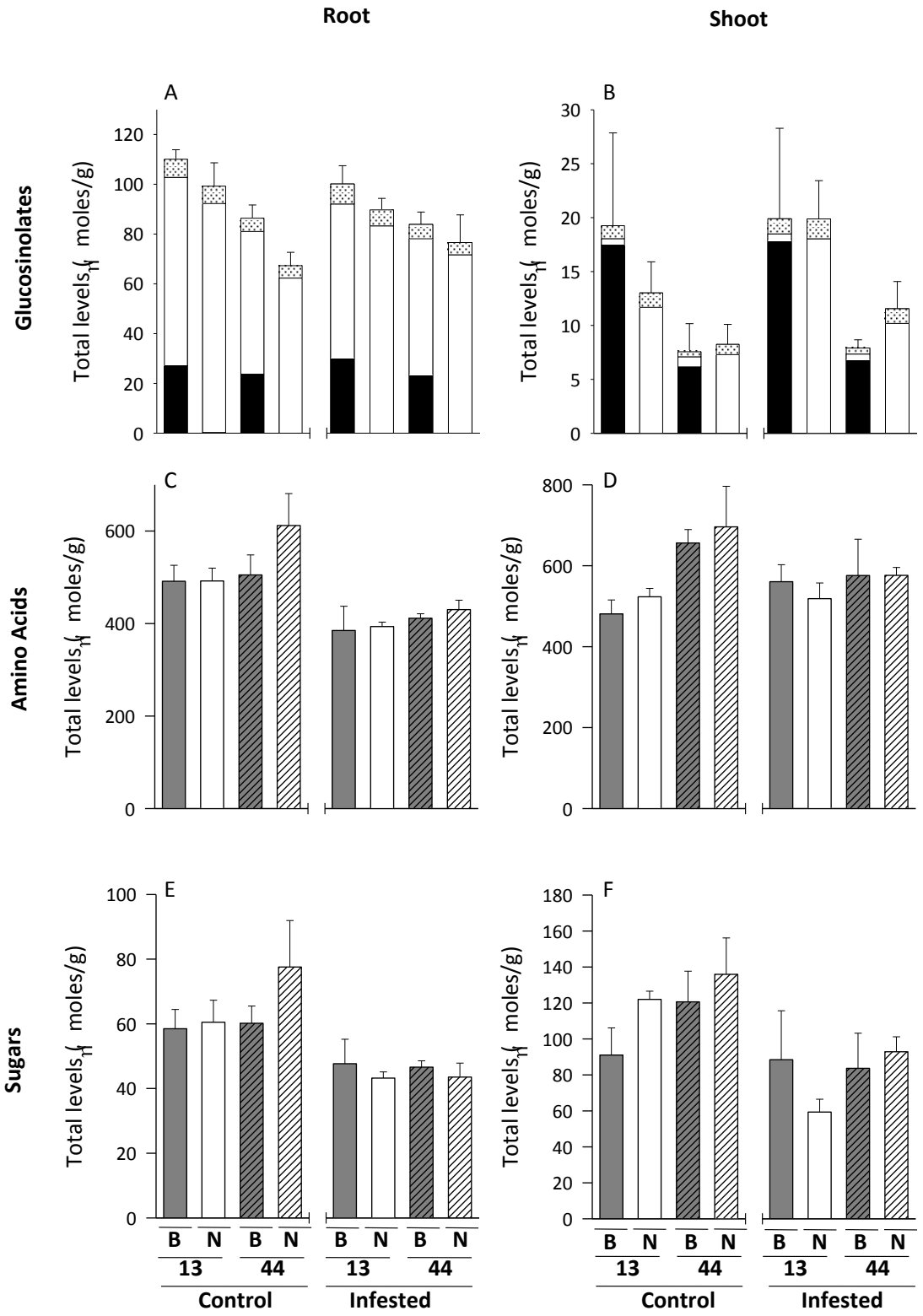

Figure 5.4 Chemical analyses of 2 families of *Barbarea vulgaris* chemotypes after 7 days of infestation with *Delia radicum.* Total glucosinolate levels subdivided for gluconasturtiin (white), glucobarbarin (black) and other glucosinolates (black dots) of roots (a) and shoots (b), total amino acid levels of roots (c) and shoots (d) and total sugar levels of roots (e) and shoots (f) (half sib family EL 13 = 13 or not hatched, EL 44 = 44 or hatched; BAR-type = B or grey bars, NAS-type = N or white bars). Error bars indicate standard errors.

Root fly infestation in an experimental garden

From 3 months after transplanting the plants in the field onwards, we found 5 up to 15 *D. radicum* pupae in the soil surrounding the plants at every harvest time (Figure 5.5). Although the soil surrounding BAR-type plants on average contained more pupae at four out of six harvests (Figure 5.5), statistical analyses revealed no significant differences in the number of pupae in the soils surrounding the two chemotypes due to the high variation in numbers of pupae·plant^{-1} (repeated measurement ANOVA over all harvests: $P > 0.05$; Kruskal Wallis tests: $P > 0.05$ for each harvest).

D. radicum infestation of plants caused flower stalks to fall over. At the end of flowering and the start of seed set in the second year after transplantation (21 July till 20 June 2006) the chemotypes had no significantly different percentage of flower stalks which had fallen over per plant (BAR: n = 196, mean: 4.91% ± 1.1, NAS: n = 198, mean 4.60% ± 1.22; Mann-Whitney *U* test: $U = 18813.00$, $Z = 0.522900$, $P = 0.601$).

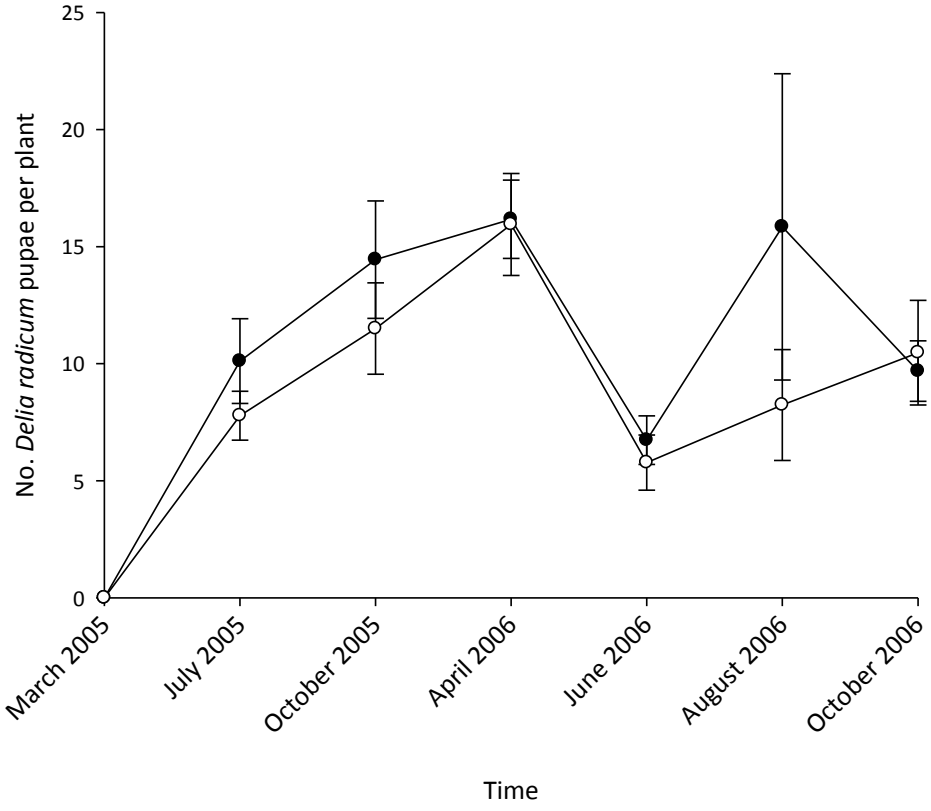

Figure 5.5 Delia infestation in an experimental garden. Numbers of *Delia radicum* pupae·plant^{-1} on *Barbarea vulgaris* chemotypes (BAR-type = black dots, NAS-type = white dots) at several time points from young rosette plants in March 2005 till late fruit ripening in October 2006.

Discussion

Effect of chemotype on root fly performance and preference

The results of the experiments testing oviposition preference, larval performance and the net result of preference and performance in the field overall suggest that BAR-type *B. vulgaris* plants were more suitable hosts for *D. radicum* than NAS-type plants. *D. radicum* larvae developed into pupae with a higher biomass when feeding on BAR-type plants compared to NAS-type plants. This effect on performance was not displayed in a higher retrieval of pupae on BAR-type plants, possibly due to an overall high mortality of the neonates. In the field experiment, there was no significant effect of chemotype on pupal retrieval, but again in most harvests more pupae were found on BAR-type plants than on NAS-type plants. The chemotypes showed no significant difference in attractiveness for oviposition. Probably, non-glucosinolate oviposition stimuli like those described by Hurter et al. (1999) are more important. However, we observed a trend towards a preference for BAR-type plants. Our findings are in line with the "mother knows best" principle (Valladares and Lawton, 1991) and the "preference-performance hypothesis" (Jaenike, 1978). The latter hypothesis predicts that when insect herbivores have offspring with limited capacity to find an alternative host plant, there is a strong selection pressure for the adult to oviposit on plants that will maximise offspring performance. The link between performance and preference of *D. radicum* on the two chemotypes shows that insect herbivores that live aboveground, but have soil-dwelling offspring, are subject to the same selection pressures. Therefore they need to be included in the preference-performance debate (Johnson et al., 2006).

Root fly infestation in an experimental garden

In an experimental garden, both chemotypes had an equal percentage of infested flower stalks and the number of retrieved pupae did not significantly differ between the chemotypes. This suggests that the performance difference under controlled conditions is either absent in the field, or overruled or countered by other interactions. For example, *D. radicum* could have been affected directly by competition with other herbivores or by attack of natural enemies, such as its predator *Aleochara* sp. (Fournet et al., 2000), its parasitoid *Trybliographa rapae* (Neveu et al., 2000) or entomopathogenic nematodes (Simser, 1992). Other organisms may also have indirectly influenced *D. radicum* by causing hydrolysis of glucosinolates, resulting in the emission of volatiles from the plants. Glucosinolate-derived plant volatiles can be detected by *D. radicum* flies and may, in addition to intact glucosinolates at the leaf surface, influence oviposition behaviour (Roessingh et al., 1992). Plant volatiles are also involved in host location of the *D. radicum* parasitoid *T. rapae*, and thus may indirectly affect performance of *D. radicum* larvae. Recently, shoot herbivory by *Pieris brassicae* was shown to negatively affect *D. radicum* and its parasitoid *T. rapae* (Soler et al., 2007a). Differences between the chemotypes in one or more of the above described interactions may have counteracted the effect on larval performance and resulting numbers of pupae as found under controlled conditions.

Effect of root fly on plant chemical composition and vice versa

In order to reveal the mechanism that causes the differential performance of root flies on the chemotypes, we analyzed plant biomass, glucosinolate, amino acid, and sugar content with and without infestation with *D. radicum*. The initial sugar and amino acid content did not differ between the chemotypes. Both chemotypes responded similarly to infestation with *D. radicum*. Under controlled conditions, all plants showed, a strong reduction of root and shoot biomass, as well as a reduction of total sugar and amino acids levels in the root. These physiological processes may weaken the plants as well as affect susceptibility to subsequent herbivory. The only chemotype specific effect was the systemic response of sugars in the shoot: NAS-type plants had a more reduced shoot sugar content compared to BAR-type plants. Therefore, the two chemotypes may experience different consequences of *D. radicum* infestation, e.g. subsequent aboveground herbivory might be affected by the small difference in shoot sugar content. It is important to notice, however, that microorganisms associated to *D. radicum* may contribute to or are affected by the observed plant responses (Lukwinski et al., 2006).

In contrast to the nutritional quality of root and shoot tissue, the glucosinolate patterns of BAR and NAS-type plants clearly differed between the chemotypes. These patterns did not change upon infestation, neither locally nor systemically. An induction as described for indole and aliphatic glucosinolates in *Brassica nigra* (Van Dam and Raaijmakers, 2006) was not observed for these phenylalanine derived glucosinolates. This absence of induction was already shown for artificial induction with jasmonic acid (Van Leur et al., 2006). As we could not detect consistent differences in amino acid or sugar content between the chemotypes, whereas the chemotypes had very different glucosinolate content (Figure 5.2), it is likely that the glucosinolate profile causes the difference in pupal biomass. Consequently, our results are the first to show that differences in glucosinolate patterns can affect *D. radicum* performance. Our findings indicate that root glucosinolates may play an important role in plant resistance against belowground herbivores. Since data on root glucosinolates patterns are scarce compared to shoot data, more research is needed to elucidate their effectiveness.

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CHAPTER 6

Interactions of *Barbarea vulgaris* glucosinolate phenotypes with above- and belowground invertebrate communities and their consequences for plant fitness

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Abstract

Herbivory exerts a continuous selection pressure on plants. One way in which plants defend themselves against herbivores, is the production of chemical defence compounds. The effectiveness of the defence, depends on the quality and quantity of these chemicals, as well as on the nature of the herbivores. Moreover, plant produced chemical defensive compounds may also influence non-target organisms, such as decomposers and their natural enemies. Here, we investigate how a chemical defence polymorphism within a plant species affects above- and belowground invertebrate communities associated with the two glucosinolate chemotypes of *Barbarea vulgaris.*

One chemotype of *B. vulgaris* contains mainly glucobarbarin (BAR-type) and the other mainly gluconasturtiin (NAS-type). The glucosinolate profiles are heritable and mainly differ qualitatively, although BAR-type plants have a slightly higher total glucosinolate level in their shoots than NAS-type plants. In the natural populations we have sampled, the chemotypes sometimes co-occurred, but the NAS-type was always present in lower frequencies than could be expected. We hypothesized that NAS-type plants, having lower levels of glucosinolates in their shoots, would be exposed to more herbivory on their shoots than BAR-type plants. Belowground, where glucosinolate levels and chemical profiles of the two chemotypes are more similar, we expect the difference to be less pronounced.

We tested our hypotheses by establishing BAR- and NAS- type plants in an experimental garden in equal frequencies and quantifying above- and belowground herbivores over two full growing seasons. Additionally, we studied the development of the entire nematode community, including bacteria-feeding, fungi-feeding and omni-carnivorous nematodes. In both seasons, the specialist *Pieris rapae* caterpillars were most abundant on shoots of NAS-type plants. On the other hand, flea beetles (*Phyllotreta spp)* and galls of *Contarinia nasturtii,* both specialist herbivores, were most abundant on BAR-type plants. Abundance of aphids (*Myzus perzicae, Brevicoryne brassicae*) and froghoppers *Cercopoidae* did not significantly differ between the chemotypes. Belowground herbivores tended to be more abundant on BAR-type plants (root flies, *Delia radicum*) or did not differ between the chemotypes (plant-feeding nematodes). Although we found a clear time resolved development of the soil nematode community, composition of this community was not affected by plant chemotype.

Despite the differential occurrence of several herbivores on the two chemotypes, plant fitness measurements (i.e. number of seed capsules, root, shoot and total biomass over time, root/shoot-ratio and rosette diameter) did not differ between the chemotypes at any harvest. Plant fitness was probably unaffected because of relatively small damage by selective herbivores. Alternatively, the opposite effects of members of the invertebrate communities may have neutralized individual effects and their consequences for plant fitness. We conclude that the under-representation of NAS-type plants in natural populations can not be simply explained by enhanced susceptibility to invertebrates.

Keywords

Defence, shoot herbivore, polymorphism, nematode community, *Delia radicum, Phyllotreta, Pieris rapae,* Brassicaceae

Introduction

Plants are under continuous selection by herbivores (Jones and Firn, 1991). In response to adaptation by herbivores, new defensive compounds with slightly different chemical structure may evolve, resulting in a so called 'co-evolutionary arms-race' (Hartmann, 1996; Pichersky and Gang, 2000). This arms-race may result in a broad variety of defence compounds within plant species. Here, we investigate whether selection pressures by above- and belowground herbivores on plants which show genetic variation in defence compounds may contribute to the maintenance of such defence polymorphisms. We use the crucifer *Barbarea vulgaris* as a model. *B. vulgaris* has heritable variation in glucosinolate content. Within natural populations, two chemotypes of *B. vulgaris* may co-occur: one containing mainly glucobarbarin (BAR-type), the other mainly containing gluconasturtiin (NAS-type). The chemotype is inherited in a Mendelian way, with a dominant allele coding for BAR-types and a recessive allele for NAS-types (Van Leur et al., 2006). The abundance of NAS-type plants in natural populations ranges from 0 up to 22%, which is less frequent than the 25% that could be expected of a recessive phenotype if the populations represented a Hardy-Weinberg equilibrium. When this underrepresentation of NAS-type plants in wild populations is due to susceptibility to herbivore attack, we expect that NAS-type plants are preferred by more herbivores than BAR-type plants. Alternatively, the NAS-type plants may simply be maintained at low frequencies in the population because of frequency dependent selection (Maynard Smith, 1989).

Glucosinolates are a well-studied example of a structurally diverse class of defence compounds that are mainly found in Brassicaceae (Fahey et al., 2001). All glucosinolates share a base skeleton of a sulphated oxime and a thioglucose moiety, but have different side chains (Halkier and Gershenzon, 2006). Upon cell rupture the glucosinolates are mixed with myrosinase, which results in the biological active hydrolysis products (Ratzka et al., 2002). Depending on the structure of the side chain, the pH at which hydrolysis takes place, and the presence of epithiospecifier protein, different breakdown products can be formed with different toxicity and volatility (Mithen, 2001a; Wittstock et al., 2003) .

The breakdown products of glucosinolates are the biologically most active compounds. The breakdown product from NAS-type plants is most likely the volatile and pungent 2-phenylethyl isothiocyanate (Chew, 1988), and that of BAR-type plants the non volatile 5-phenyloxazolidine-2-thione (Kjaer and Gmelin, 1957). The 2-phenylethyl isothiocyanate is toxic or deterrent to a broad range of organisms, such as fungi (Tierens et al., 2001), nematodes (Potter et al., 1999; Serra et al., 2002), snails (Kerfoot et al., 1998), insect herbivores (Wittstock et al., 2003) and other plants (Yamane et al., 1992). Additionally, isothiocyanates are involved in indirect defence by attracting insect parasitoids and parasites (Wittstock et al., 2003). On the other hand, a number of specialist herbivores like *Pieris rapae*, *Delia radicum*, and *Phyllotreta nemorum* are attracted to volatile isothiocyanates as

well, and use the intact glucosinolates as oviposition cues (Chew, 1988; Nielsen, 1997; Städler et al., 2002). Oxazolidine-thiones, to which the BAR-type product belongs, inhibit infection with the soil fungus *Plasmodiophora brassicae* (Ludwig-Müller et al., 1999) and have strong anti-nutritional effects on mammals (Fenwick et al., 1983).

Due to the qualitative differences in glucosinolates and breakdown products, the *B. vulgaris* chemotypes are likely to have different effects on above and belowground invertebrate communities associated with these plants. In previous studies, we showed that under controlled conditions the aboveground generalist herbivore *Mamestra brassicae* performed better on NAS-type plants and had a high mortality on BAR-type plants (Chapter 4). On the other hand, the belowground specialist *Delia radicum* performed slightly better on BAR-type plants (Chapter 5), whereas the preference and performance of the aboveground crucifer specialist *Pieris rapae* did not differ between the chemotypes (Chapter 4). These results obtained under greenhouse conditions suggest that none of the chemotypes is better defended in the absolute sense, and this makes it difficult to predict which chemotype suffers the most herbivory in natural populations.

In this study, we test the hypothesis that NAS-type *B. vulgaris* plants are attacked by more herbivores than BAR-type plants. Because differences between BAR-type and NAS-type glucosinolate profiles are smaller in roots than in shoots (Van Leur et al., 2006), our specific hypothesis is that larger chemotype effects will be found on the aboveground than on the belowground invertebrate community. We planted BAR-type and NAS-type plants in an experimental garden in equal densities to rule out frequency dependent selection processes to occur, and quantified aboveground herbivory in weekly censuses during two consecutive growing seasons. Belowground, we investigated the abundance of root-feeding *D. radicum* and infestation of endoparasitic and ectoparasitic plant feeding nematodes.

Once glucosinolates or their breakdown products are released into the soil from root exudates or the decay of plant organs, they may have important effects on a broad range of non-target organisms in the rhizosphere (Brabban and Edwards, 1995; O'Callaghan et al., 2000; Tierens et al., 2001; Halkier and Gershenzon, 2006). For example, the dominant fungal species in soil near glucosinolate containing Brassicaceae are different than the dominant fungal species found elsewhere, and show increased tolerance to isothiocyanates (Ishimoto et al., 2000). Therefore, we also included bacterial-feeding, fungal-feeding, and omni-carnivore nematodes in our study.

Methods

Plant material

Barbarea vulgaris is native in Eurasia and introduced to North America, Africa, and Australia where it is a noxious weed. It grows mainly in grassy vegetations along roads, rivers and ditches. *B. vulgaris* is a short lived perennial plant that forms a rosette in the first year, and produces one or more flowering stalks in the second year (Hegi, 1962). To obtain plants from various genetic backgrounds, we collected seeds from three natural populations of *B. vulgaris* in the Netherlands: Elderveld (EL, 51.95 °N; 5.87 °E) , Heteren (HE, 51.95 °N; 5.77 °E) and Oosterbeek (OO,51.98 °N; 5.85 °E) (Van Leur et al., 2006). From the HE population we used seeds from one NAS-type maternal plant which had been freely cross-pollinating in the population. The EL and OO seeds were derived from two reciprocal crosses of a NAS-type and a heterozygous BAR-type parent from the same population. Of each cross 300 seeds were grown and the first fully grown leaves were sampled to determine the glucosinolate type. To obtain 100 BAR-type and 100 NAS-type plants per population origin we multiplied 50 BAR-type and 50 NAS-type plants of the EL and OO crosses by cutting 6-weeks-old plants in two halves and regrowing them in separate pots. As we had only 25 NAS-type plants from the HE seed batch we decided to multiply 25 BAR-type and 25 NAS-type 6-weeks-old plants by cutting the plants in two halves, growing them in separate pots and repeating that procedure with 9-weeks-old plants. Four weeks prior to planting in the field, 600 12-weeks-old plants were placed in a half-open greenhouse (min. temperature 4 °C) to gently acclimatize the plants to outdoor conditions.

Glucosinolate type determination

To determine the chemotype of each individual, the youngest fully grown leaf was sampled and frozen at -20 °C, lyophilized and stored at -20 °C until analysis. Glucosinolates were extracted from ground samples with 70% MeOH, desulphated with arylsulphatase (Sigma, St. Louis, IL, USA) on a DEAE-Sephadex A 25 column (EC, 1990) and separated on a reversed phase C-18 column on HPLC with a CH_3CN–H_2O gradient as described by van Dam and Raaijmakers (2006). Glucosinolate analysis was performed with a PDA detector (200–350 nm) with 229 nm as the integration wavelength. Desulfoglucosinolate peaks were identified by comparison of HPLC retention times and UV spectra with authentic standards isolated from *B. vulgaris* as described previously Agerbirk et al. (2001b), as well as with standards provided by M. Reichelt, MPI Chemical Ecology, and a certified rape seed standard (Community Bureau of Reference, Brussels, code BCR-367R). For glucobarbarin, we used the same response factor as for gluconasturtiin. When the peak area of glucobarbarin divided by the peak area of gluconasturtiin was > 10, the plant was considered a BAR-type, when this ratio was < 0.1 it was considered a NAS-type. The < 1% of the samples that could not be classified clearly as either type was omitted from the experiments.

Set-up common garden experiment

Plants were placed in a common garden at the NIOO-KNAW Centre for Terrestrial Ecology in Heteren, The Netherlands in April 2005. The vicinity of natural populations of crucifers (distance to natural *B. vulgaris* population HE < 2 km) and a history of cultivating several

Brassica spp. in nearby garden plots ensured the presence of crucifer specialists. The plot had been covered with root-cloth for four years to reduce the establishments of weeds. Two weeks prior to the start of the experiment the upper 20 cm of the soil was fraised to facilitate planting and fertilized with 5.75 g $Ca(H_2PO_4)_2$ m^{-2}, 12.5 g $K_2SO_4.MgSO_4$ m^{-2}, 15.0 g $MgSO_4$ m^{-2} and 6.25 g $(NH_4)_2SO_4$ m^{-2}. The plants were planted in a 12 × 13 m^2 area at 0.5 m distance from each other. As a result, the design consisted of 25 rows with 24 plants per row, forming lanes with BAR-type and NAS-type plants in alternation; population origins were randomized. Throughout the experiment the experimental, the area was weeded manually at regular intervals to eliminate competition with other plants.

Assessing aboveground and belowground invertebrate herbivore communities

Every week, between March and November, five rows were inspected according to a latin square design, starting with row 1, 6, 11, 16, 21 in the first week. Thus, individual plants were inspected every 5 weeks. We quantified herbivores (adults or eggs·$plant^{-1}$) of the species which are known to feed on *Brassica olearacea* (Mitchell et. al. (1979) (Table 6.3), and added other herbivore species which we observed on > 50% of the plants at one census date.

As *Phyllotreta spp.* beetles are highly mobile and easily disturbed, the damage to the plants by foraging *Phyllotreta spp.* beetles was quantified as the amount of fresh holes in all leaves of a plant, expressed as a number between 0 (no damage) and 100 (all leaves heavily damaged). The first year, we monitored the presence of aphids (*Myzus persicae* and *Brevicoryne brassicae*) as binary data. In the second year, we counted the number of aphids when < 100 per plant and estimated the number when > 100 per plant. The second year, we determined plant performance by measuring the rosette diameter (between April-May) and monitored whether plants were flowering.

Harvests

Destructive harvests were made in April (t1), July (t2) and October (t3) 2005 and in April (t4), June (t5), August (t6) and October (t7) 2006. At each time point we harvested six plants per population origin (3) × chemotype (2) = 36 plants per harvest. These 36 plants were collected from random positions. Prior to plant excavation, we collected soil cores of 15 cm deep and 2.5 cm diameter to examine the nematode community. To ensure that major and minor roots were included and to prevent sampling roots from neighbouring plants, we took two cores directly adjoining and two cores at 5 cm distance from the main root. Soil cores were bulked per plant and nematodes were extracted using Oostenbrink elutriators (Oostenbrink, 1960). For each soil sample, nematodes were extracted from the roots using a mist chamber. The total number of nematodes was counted in 10% of the total extracted soil volume. Nematode samples were heat-killed and fixated in 35% formaldehyde diluted to 4%. Nematodes were determined to genus or family level according to Bongers (1988) using an inverted light microscope (10 × 20), and were allocated to feeding groups according to Yeates et al. (1993). Due to extreme turbidity of the nematode samples from harvest 4, this date was excluded from our analyses.

After the soil cores were taken, the shoots were cut at the shoot-root interface and placed in a paper bag. The roots were excavated by digging out a fixed volume of soil surrounding the roots (20 × 20 × 20 cm^3), resulting in approximately 7 kg soil/root mass. These soil/root samples were stored in plastic bags at 4˚C for a maximum of three days. The roots were retrieved by washing out soil with ample water over a sieve in order to collect *D. radicum* pupae. A subset of the pupae was reared to adult and the emerging flies were confirmed to be *D. radicum* by Y. Jongema of the Laboratory of Entomology, Wageningen University, The Netherlands. Plant dry mass was determined by drying roots and shoots separately at 70 °C to constant weight and weighing them on a balance to the nearest 0.1 g.

Statistical analyses of herbivores densities

Statistical analyses were performed with STATISTICA (data analysis software system), version 8.0 (StatSoft, Inc., Tusla, OK, USA). Normality and homogeneity of variances were checked by inspection of the residuals and, when the data did not meet the proper assumptions, they were log-transformed or non-parametrically tested.

The periodical removal of plants resulted in an unbalanced dataset for the weekly observations, which was circumvented by grouping data per chemotype of plants in four adjacent lanes. Consequently we could not analyse population origin as a separate factor in our analyses. Having 6 × 4 = 24 lanes, all plants in the field were assigned either to one of the six BAR-type groups or one of the six NAS-type groups. Thus six replicate measurements of each chemotype per time point were obtained. The field data for number of retrieved pupae and the percentage of infested flower stalks did not fit normal distributions and were therefore tested with a non-parametrical Kruskal-Wallis test and a Mann-Whitney U test, respectively. Plant root, shoot and total dry mass, as well as the shoot/root-ratio were analysed by factorial ANOVA's with harvest and chemotype as factors. In contrast to the community analysis of the nematodes (see below) the aboveground herbivore load was statistically analyzed for each highly abundant species (++ in Table 6.1) separately. Rare species (+ in Table 6.1) were not analysed. The herbivore species peaked at different periods in time, which made it difficult to directly compare the abundances of all herbivores on each plant chemotype over the entire observation period. To facilitate this comparison, the abundance of each herbivore, was summed over the two years of the experiment. These cumulative values were used as indicator of the abundance with which the herbivores were found on each chemotype. A visual representation of the proportional preference/occurrence of a herbivore on NAS-type plants compared to BAR-type plants of each herbivore species was calculated as (Relative preference = 100 - (100 × average NAS / average BAR) (Figure 6.1).

Multivariate analyses of nematode data

Nematode community composition was analyzed using multivariate ordination techniques. Analyses were performed for all taxa together, for plant feeders (totally and separately for endoparasitic and for ectoparasitic plant feeders), bacterial feeders, fungal feeders, and omni-carnivores (De Deyn et al., 2004; Kardol et al., 2005). For each data set, we performed detrended correspondence analysis (DCA) in order to decide whether a linear or unimodal

type of ordination method may be a better choice. For all taxa together, endoparasitic plant feeders, ectoparasitic plant feeders, bacterial feeders, and fungal feeders, the longest DCA gradient was lower than 4, indicating that linear methods would meet. For total plant feeders and omni-carnivores, the longest DCA gradient was higher than 4, indicating that unimodal methods would be most appropriate (Lepš and Šmilauer, 2003). Results from linear and unimodal analyses were highly comparable and did not differ qualitatively. Therefore, we decided to apply linear ordination methods to all data sets, to enhance consistency in analyses.

First, we analyzed the data (all nematode taxa together) using principal component analysis (PCA). PCA showed no separation based on plant population origin (Heteren, Elderveld, Oosterbeek) and, therefore, population origin was not included in further analyses. Second, for each data set, we tested the explanatory power of chemotype, time and their interaction term (i.e. chemotype × time) using redundancy analysis (RDA).). RDA was performed for nematode feeding group composition. The partial effect of plant chemotype (BAR, NAS) was tested including time as covariable. Similarly, the partial effect of time was tested including plant chemotype as covariable. Significance was tested using Monte Carlo Permutation tests (999 permutations) restricted for block design with blocks defined by covariables, i.e. time and plant chemotype, respectively. The interaction term plant chemotype × time was tested using unrestricted permutations. Additionally, we performed RDA for each time point separately and tested the effect of chemotype, using unrestricted Monte Carlo Permutation tests (999 permutations). In all RDA's we included dry mass of roots as covariate. Multivariate analyses were performed using log-transformed nematode abundance data. Analyses were performed using CANOCO version 4.5 (Ter Braak and Šmilauer, 2002).

Results

Aboveground herbivores

The aboveground herbivore community in the experimental garden consisted of leaf chewing and sap-sucking herbivores. Generalists and crucifer specialists were found of both feeding guilds. Except *Plutella xylostella* and *Evergestis forficalis*, we detected all herbivores that were described to be present on *Brassica olearacea* by Mitchell et al. (1979) (Table 6.1). In the second year, when plants flowered, we additionally found generalist froghoppers (*Cercopoidea*) on more than 50% of the plants. Both on BAR and NAS-type plants, visible feeding damage to shoots was predominantly caused by *Pieris rapae* larvae and *Phyllotreta sp* adults.

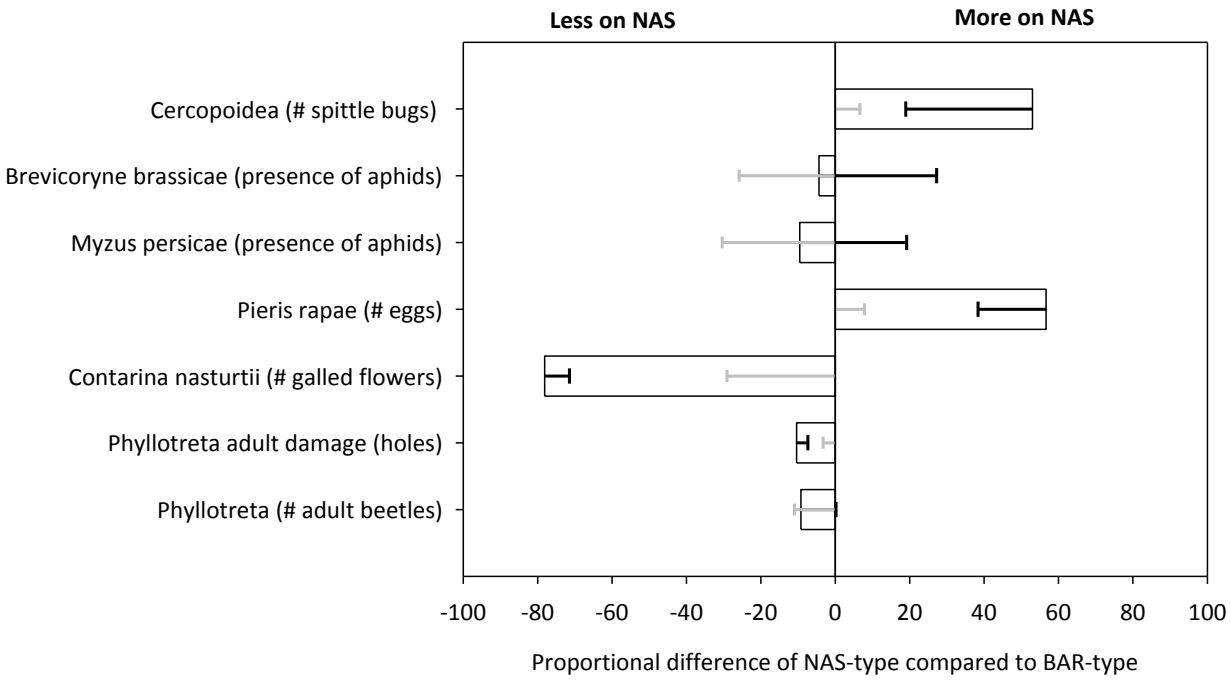

Figure 6.1 Comparison of herbivore preferences between *Barbarea vulgaris* chemotypes grown in an experimental garden. Values are proportional, cumulated per plant over 2 years of monitoring. The relative preference was calculated as = 100-(100 * average NAS/average BAR). Error bars indicate the SE (grey = SE of BAR-type value; black = SE on NAS-type value).

Table 6.1 Abundance of herbivores, known to damage *Brassica oleracea* (Mitchell et al., 1979), on *Barbarea vulgaris* (- not found, + means that the presence did never exceed 50% of the plants, ++ means that the presence > 50% of the plants at one time point minimally).

Order	Family	Species	Common name	On *B. vulgaris*
Hemiptera	Aphididae	*Brevicoryne brassicae*	Cabbage aphid	++
		Myzus persicae	Green peach aphid	++
	Aleyrodidae	*Aleyrodes proletella*	Cabbage whitefly	+
Lepidoptera	Pieridae	*Pieris rapae L.*	Cabbage white	++
		Pieris brassicae	Large white	+
	Plutellidae	*Plutella xylostella (L.)*	Diamondback moth	-
	Pyralidae	*Evergestis forficalis*	Garden pebble moth	-
	Noctuidae	*Mamestra brassicae (L.)*	Cabbage moth	+
		Autographa gamma	Silver-Y moth	+
	Tortricidae	*Clepsis spectrana*	Cabbage leafroller	+
Coleoptera	Chrysomelidae	*Phyllotreta nemorum/undulata*	Flea beetle	++
Thysanoptera	Thripidae	*Thrips tabaci Lind.*	Onion thrips	+
Diptera	Anthomyiidae	*Delia radicum* (L.)	Cabbage root fly	++
	Cecidomyiidae	*Contarinia nasturtii*	Swede midge	++

Over the two years of observation, there were significantly more *Pieris rapae* eggs found on NAS-type plants (11.3 eggs·plant^{-1}) than on BAR-type plants (7.2 eggs·plant^{-1}; t-test: t = 2.85, df = 10, *P* = 0.017; Figure 6.1, Figure 6.2a.). In both years, egg abundance peaked in September (Figure 6.2a). In contrast to *P. rapae,* more damage of *Phyllotreta spp* beetles was found on BAR-type plants. The most numerous *Phyllotreta spp* observed were the crucifer specialists *Phyllotreta undulata* and *Phyllotreta nemorum.* The quantity of feeding damage over time shows that *Phyllotreta* beetles were present in both years and although both chemotypes were damaged, BAR-type plants had significantly more holes than NAS-type plants (t-test: t = -2.31, df = 10, *P* = 0.043; Figure 6.2b). In line with this, we found more adult beetles on BAR-type plants, however, this was not statistically significant. The presence of adult beetles was considered a worse estimate of the *Phyllotreta spp* preference than the leaf damages, because the beetles escaped when the observer was approaching the plant.

BAR-type plants also had more galls of the Cabbage gall midge *Contarinia nasturtii* (Man-Whitney *U* test: *Z* = -2.24, df = 10, *P* = 0.025; Figure 6.1.). These flower-bud galls were only found from May till June in the second year, when the plants were flowering (Figure 6.2c). Another herbivore which was only found on inflorescences was the froghopper (*Cercopoidea*). Despite a higher abundance on NAS-type plants (average no. of froghoppers·plant^{-1} on BAR-type 2.39 and on NAS-type 3.66), the overall abundance of this sap-sucking herbivore did not differ between the chemotypes (t-test: t = 1.52, df = 10, *P* = 0.157).

The specialist aphid *Brevicoryne brassicae* did not differ in abundance between the two chemotypes, nor did the generalist aphid *Myzus persicae*. *M. persicae* was mainly present on fresh leaves in the spring, while *B. brassicae* was mainly found on senescing leaves in the fall. At each harvest time, we found 5 - 15 *D. radicum* pupae in the soil surrounding the plants. Although the soil surrounding BAR-type plants on average tended to contain more pupae at four out of six harvests, statistical analyses revealed no significant differences between the two chemotypes (repeated measurement ANOVA over all harvests: P > 0.05; Kruskal-Wallis tests: P > 0.05 for each harvest). The belowground herbivore *Delia radicum* alone caused more damage to the plants than the aboveground herbivores together. Due to tunneling of the larvae in the main roots, some plants dehydrated and in some cases even died. At the end of flowering and the start of seed set in the second year (21 June till 20 July 2006) the chemotypes showed similar percentages of fallen flower stalks·plant^{-1} due to *D. radicum* feeding (BAR: n = 196, mean: 4.91% ± 1.1, NAS: n = 198, mean 4.60% ± 1.22; Mann-Whitney *U* test: *U* = 18813.00, *Z* = 0.522900, *P* = 0.601).

In addition to the most frequently found herbivores above, we infrequently observed the following herbivore species: slugs, pollen feeding beetles, some *Curculionoidea*, root feeding *Agrotis* larvae. Several natural enemies of the herbivores were observed as well, such as lady beetles (eggs, larvae and adults), *Chrysopidae* (eggs, larvae and adults), a predator and parasitoid of the root flies *Aleochara bilineata*, parasitized aphids of *Myzus persicae* (mummies) and some larvae of *Pieris rapae* and *Pieris brassicae* were parasitized by *Cotesia glomerata* and *Cotesia rubecula*. However, the numbers of recorded natural enemies were too low for statistical analysis.

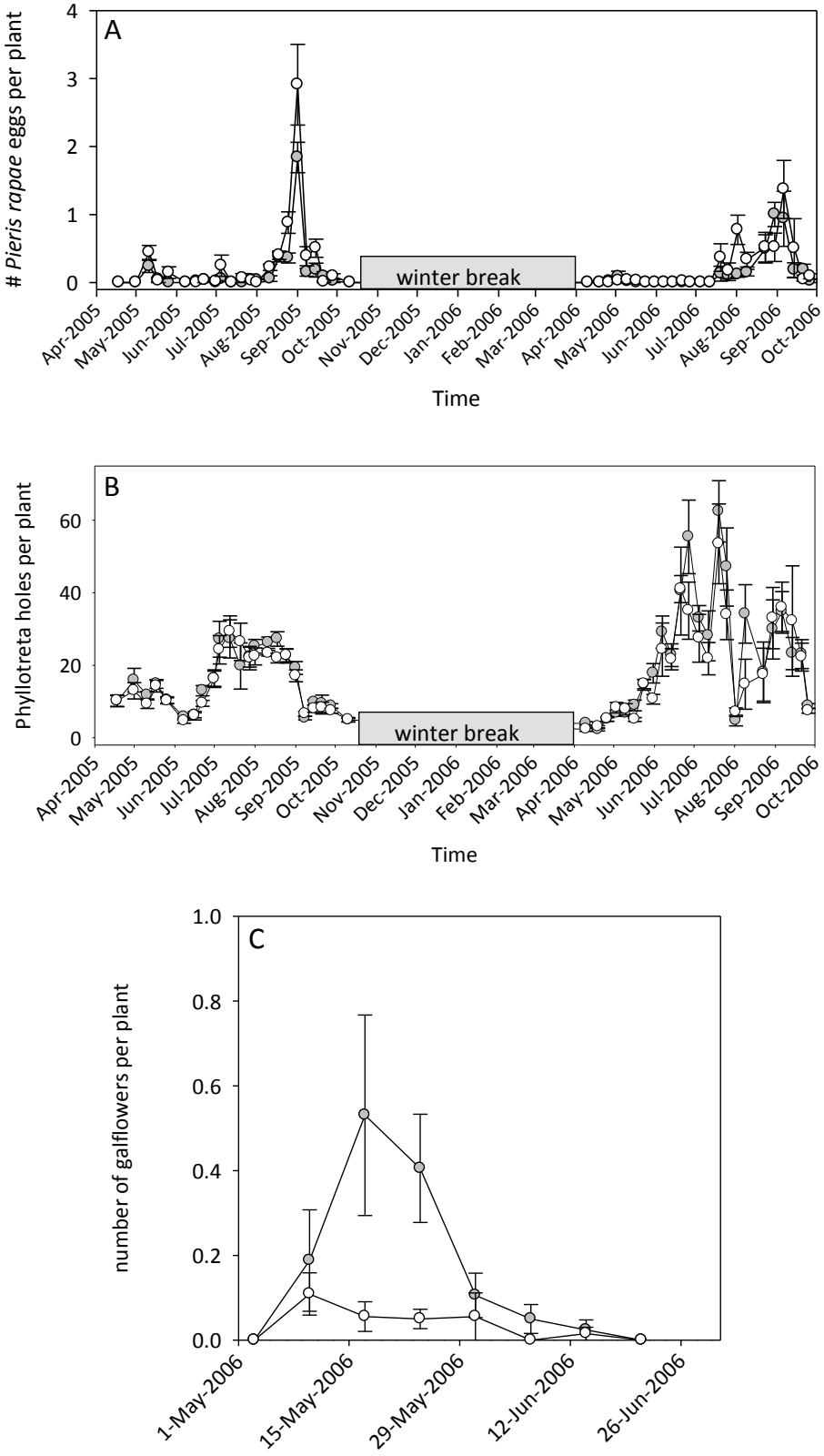

Figure 6.2 Abundance of (A) *Pieris rapae* eggs, (B) *Phyllotreta spp. d*amage, (C) number of gall flowers by *Contarinia nasturtii* on BAR-type (grey circles) and NAS-type (white circles) plants in an experimental garden measured over time. Error bars indicate the SE.

Nematodes

Nematode abundance increased over time, probably as a result of plant colonization and multiplication (Figure 6.3). There was a significant time effect on the nematode community for all taxa together, for each feeding group, as well as for the feeding group composition (Table 6.2). At the start of the experiment (harvest 1) hardly any nematodes were present. The abundance of nematodes gradually increased during the first year (Figure 6.3). The second year, when plants were reproducing, the samples from the different harvests showed stronger overlap (see envelopes in Figure 6.3). The most abundant taxa in both chemotypes were the ectoparasitic plant feeder Criconematidae, the bacterial feeding Cephalobidae and Rhabditidae, and the fungal feeding *Aphelenchus* (Table 6.3).

Table 6.2 Results of redundancy analysis (RDA) testing the effect of plant chemotype (NAS, BAR) and time (t1-3, t5-7) on the composition of the soil nematode community. F- and P-values are based on Monte Carlo permutation tests (**P* < 0.05,***P* < 0.001).

	F			Explained variance (%)		
	Type	**Time**	**Type × Time**	**Type**	**Time**	**Type × Time**
All taxa	0.83	111**	1.08	0.2	32.8	0.3
Plant feeders (total)	0.96	33.1**	1.06	0.5	17.7	0.6
- Endoparasites	0.62	21.7**	0.44	0.6	19.5	0.4
- Ectoparasites / root hair feeders	1.22	26.3**	1.66	0.7	15.5	1.0
Bacterial feeders	0.76	106**	1.19	0.2	31.5	0.4
Fungal feeders	0.83	119**	0.68	0.3	40.0	0.2
Omni-carnivores	0.45	52.4**	0.41	0.2	22.6	0.2
Feeding group composition	1.14	230**	0.18	0.2	47.3	0.0

Plant chemotypes did not separate in the principle component analysis (Figure 6.3). Moreover, the amount of variation in nematode community composition (total and within feeding groups) and in feeding group composition that could be explained by plant chemotype was very low and not significant when tested in redundancy analysis (Table 6.2). The interaction of chemotype and time was not significant either. Moreover, when analyzed within time points, we never found a chemotype effect on nematode community composition (for all taxa together, within feeding groups, or for feeding group composition) (data not shown).

Table 6.3 Average number (and SE) of nematodes·100g^{-1} dry soil in the root/soil sample of *Barbarea vulgaris* chemotypes over the three harvests in the second year of the garden experiment (n = 54).

	BAR-Type		NAS-Type	
Taxon	**Average**	**SE**	**Average**	**SE**
Plant feeders	**1568**	**290**	**1922**	**379**
Endoparasites	*196*	*37.9*	*413*	*224*
Rotylenchus	136	34.4	145	36.7
Heterodera	16.3	15.1	1.32	1.32
Meloidogyne	0.00	0.00	224	224
Helicotylenchus	2.18	2.18	0.00	0.00
Pratylenchus	0.00	0.00	0.58	0.58
Ectoparasites/root hair feeders	*1413*	*288*	*1551*	*319*
Criconematidae	1336	286	1465	320
Trichodoridae	19.5	7.14	27.9	9.37
Dolichodoridae	37.5	11.8	21.5	7.36
Malenchus	16.0	5.90	9.49	5.68
Tylenchidae	3.02	1.33	7.65	4.75
Longidorus	1.47	0.90	0.36	0.36
Paratylenchus	0.00	0.00	18.5	18.5
Bacterial feeders	**5431**	**681**	**5390**	**818**
Cephalobidae	3181	453	3432	510
Rhabditidae	1093	310	771	347
Wilsonema	839	159	816	153
Acrobeles	138	413	176	38.5
Plectidae	139	23.6	166	31.1
Teratocephalus	25.0	6.99	14.4	4.37
Alaimus	12.9	3.00	12.8	4.05
Bunonema	0.34	0.34	0.00	0.00
Fungal feeders	**3758**	**928**	**2933**	**527**
Aphelenchus	3722	927	2863	525
Aphelenchoides	36.22	8.36	70.1	15.2
Omni-carnivores	**529**	**46.7**	**536**	**52.6**
Dorylaimidae	251	37.1	235	37.6
Prismatolaimus	229	32.8	256	44.7
Mononchidae	47.7	13.3	43.1	9.64
Monhysteridae	1.62	0.93	1.82	1.04
Total	**11288**	**1419**	**10782**	**1252**

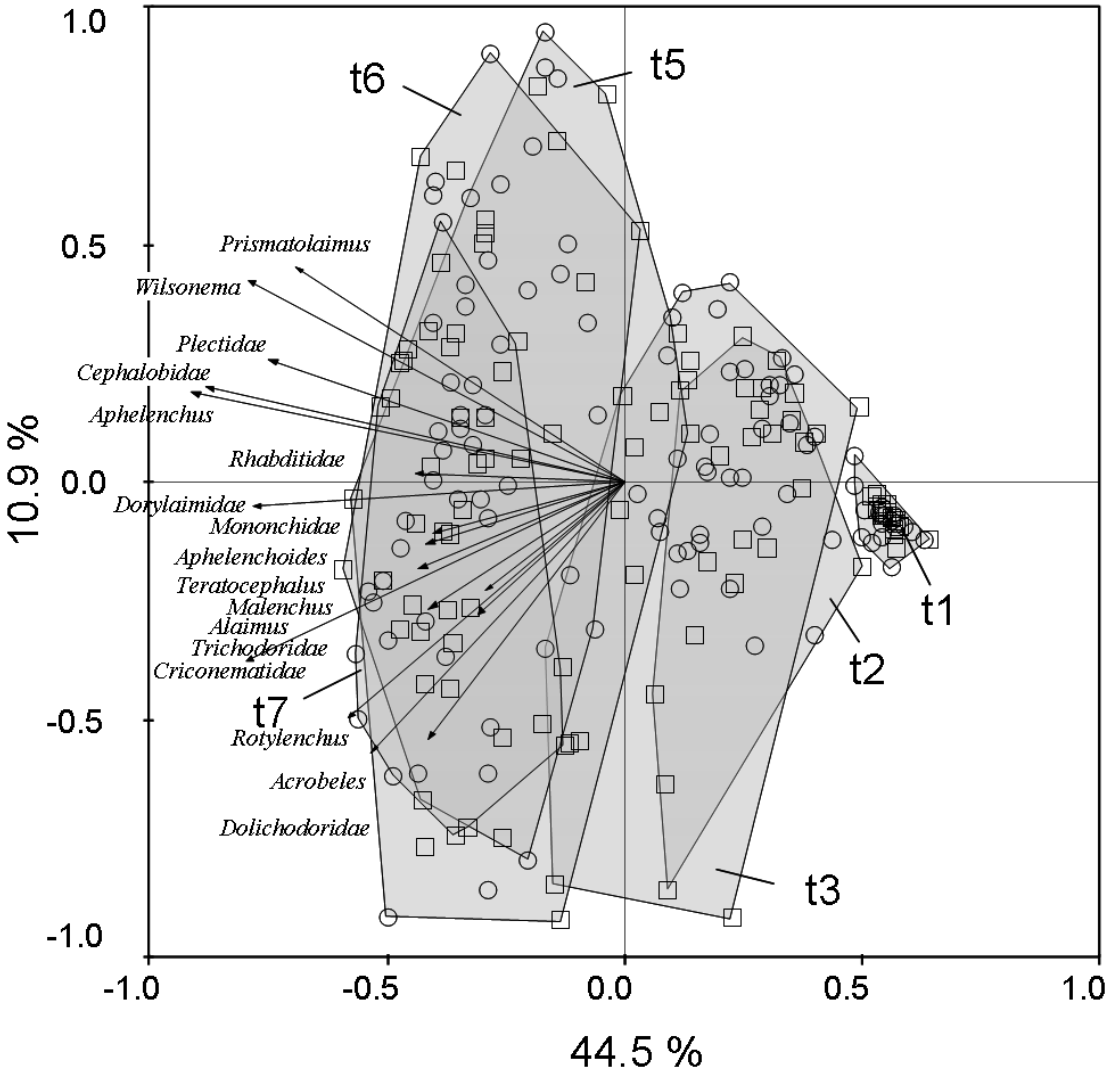

Figure 6.3 Species-sample plot of principal component analysis (PCA) of nematode taxon composition. Envelops are drawn around samples from the same harvest time. For clarity, only taxa with a fit range ≥ 10 are included. Percentages along the axes correspond to the amount of explained variability in taxon composition. ☐ = NAS-type plant, ○ = BAR-type plant.

Plant fitness

During the experiment the plant biomass increased, but there were no differences between the chemotypes (factorial ANOVAs on root, shoot and total dry mass: type effect, $F_{1,205} < 1.62$, $P > 0.05$; time effect, $F_{5,205} > 8.83$, $P < 0.0001$). The shoot/root ratio also increased over time, but no differences between the chemotypes were found (Factorial ANOVAs on shoot/root-ratio: type effect, $F_{1,205} = 0.15$, $P > 0.05$; time effect, $F_{5,205} = 3.78$, $P < 0.05$). In line with the above, the increase in rosette diameter was similar for both chemotypes (factorial ANOVA on rosette diameter: type effect: $F_{1,428} = 1.23$, $P > 0.05$; time effect $F_{4,428} = 7.40$, $P < 0.001$). The first plants of both chemotypes started flowering end of April in the second year of the experiment. The third week of May, all plants were flowering, irrespective of the chemotype. At the end of the flowering period, the chemotypes had similar numbers of seed capsules (BAR-type on average 2423 and NAS-type on average 2432 capsules; t-test: $t = 0.024$, $P = 0.981$, $df = 35$).

Discussion

Based on the lower concentrations of glucosinolates and the lower frequencies of NAS-type compared to BAR-type plants in *Barbarea vulgaris* population, we hypothesized that NAS-type plants would be colonized by more invertebrate herbivores than BAR-type plants. Indeed *Pieris rapae* eggs were more abundant on NAS-type plants. However, *Phyllotreta sp* and *Contarinia nasturtii* were more abundant on BAR-type plants. The abundance of aphids, root flies, froghoppers and nematodes did not differ between the chemotypes. Also the nematode community composition was not affected by plant chemotype.

Effects on Phyllotreta

Phyllotreta undulata and *Phyllotreta nemorum* are both crucifer specialist and known to respond to glucosinolates (Nielsen, 1978; Hopkins et al., 1998; Liblikas et al., 2003). Collectively, these two species caused more damage to BAR-type plants than to NAS-type plants. Differential susceptibility to *P. nemorum* was also shown for two other *B. vulgaris* types (P-type and G-type) in a Danish polymorphism (Agerbirk et al., 2001a). Different from our BAR/NAS polymorphism, the P-type and G-type also differed in trichome density, did not co-occur in natural populations, were hard to cross, and neither of the Danish types had a high content of gluconasturtiin in the leaves (N. Agerbirk, personal communication). Therefore, we cannot directly compare the *Phyllotreta spp*. susceptibility in our BAR/NAS polymorphism with that of the Danish polymorphism.

Field versus greenhouse conditions

Differences in numbers of *P. rapae* eggs between BAR-type and NAS-type plants in the experimental garden do not correspond with previous greenhouse experiments, where *P. rapae* did not show differential preference or performance on the chemotypes (Chapter 4). A possible explanation for this inconsistency might be that, in the garden experiment, the abundance of a certain herbivore species on a chemotype did not only represent the preference and performance of that herbivore, but is the net result of bottom-up (plant quality) and top-down (control by natural enemies) effects. Moreover, indirect effects of the environment on plant chemistry may have altered plant-herbivore interactions. In contrast to the intact plants used in the greenhouse experiment, plants under field conditions are likely to be damaged by other herbivores. This damage may lead to higher levels of glucosinolate breakdown products which may deter generalist or attract specialist herbivores and their natural enemies (Wittstock et al., 2003).

In addition to the immediate plant response, herbivory may alter plant chemistry on a longer term, e.g., by induction of secondary defence compounds, and thereby negatively affect subsequent herbivores (Agrawal, 1999; Van Dam et al., 2004; Bruinsma et al., 2007) and even higher trophic level organisms (Soler et al., 2005; Soler et al., 2007a). Previous experiments showed that the total glucosinolate levels of both *B. vulgaris* chemotypes could be induced by application of jasmonic acid to roots or shoots, and that this effect was stronger for NAS-type shoots than for BAR-type shoots. Although root herbivory in Chinese cabbage induced gluconasturtiin levels with 58% (De Villena et al., 2007), we did not find

higher root or shoot glucosinolate levels in *B. vulgaris* when infested belowground with Cabbage root fly larvae (*Delia radicum)* (Chapter 5).

Aboveground versus belowground

Based on smaller glucosinolate differences in roots compared to shoots, we hypothesised smaller chemotype effects on the belowground than on the aboveground invertebrate community. Root flies tended to be more abundant on BAR-type plants. In line with our hypothesis, the chemotypes did not differ in infestation of plant-parastitic nematodes. Also, the nematode taxon and feeding group composition did not differ between BAR- and NAS-type plants. Plant species and plant functional groups can strongly differ in abundance and composition of their associated plant feeding nematodes, as well as in abundance of associated bacterial-feeding, fungal-feeding and omni-carnivorous nematodes (De Deyn et al., 2004; Viketoft et al., 2005). However, we did not find such differences between different chemotypes within a plant species. Isothiocyanates have been shown to be toxic to plant parasitic nematodes and *Brassicacea* are used in biofumigation to reduce nematode levels in the soil (Brown and Morra, 1997; Potter et al., 1998; Lazzeri et al., 2004; Zasada and Ferris, 2004; Rahman and Somers, 2005). Nevertheless, in our study the slightly higher levels of the isothiocyanate forming gluconasturtiin in NAS-type plants did not result in a lower abundance of ecto- or endoparasitic nematodes. Possibly, the less studied oxazolidine-2-thiones formed in BAR-type plants also have nematicidal activity.

Alternatively, the absence of chemotype effects on soil nematodes could be due to the temporal dynamics in the nematode community. We established our experiment in an almost nematode free soil, and the nematode community strongly changed over time. Other studies on similar soil types showed that nematode community development in newly established plant communities, is a longer-term process, and during the initial stages of development, the soil nematode community may develop rather independent of the plant community (Korthals et al., 2001; Kardol et al., 2005). Therefore, the nematode community of well-established populations of *B. vulgaris* may differ from the nematode community observed in our two-year garden experiment. Possibly, the analyses of the nematode community of an established natural population would reveal differences between BAR-type and NAS-type plants.

Plant fitness

Plant fitness measurements (number of seed capsules, root, shoot and total biomass over time, root/shoot ratio over time and rosette diameter) did not differ between the chemotypes, despite the differential effects of several herbivores. This suggests that the herbivory was not severe enough to affect plant fitness. On the other hand, some herbivores were more abundant on NAS-type and others on BAR-type plants, which may provide counteracting effects. In our experimental design we reduced interspecific plant competition by planting the plants at 0.5m distance, and we eliminated effects of intra-specific plant competition by weeding. However, in natural populations where inter- and intra-specific competition is present, plants may be more stressed and the effect of herbivory in plant fitness may be enlarged.

Conclusions

We conclude that the under-representation of NAS-type plants in natural populations can not be simply explained by enhanced exposure to invertebrate herbivores but is of a much more complex nature. As different herbivore species showed opposite preferences, there may be an ecological trade-off in which being better defended against some herbivores, comes with the costs of a higher susceptibility to other herbivores. In addition, other processes such as frequency dependent selection may play a role. Therefore, the local composition of the herbivore community in a natural population can determine, or be determined by, the frequencies of the two chemotypes.

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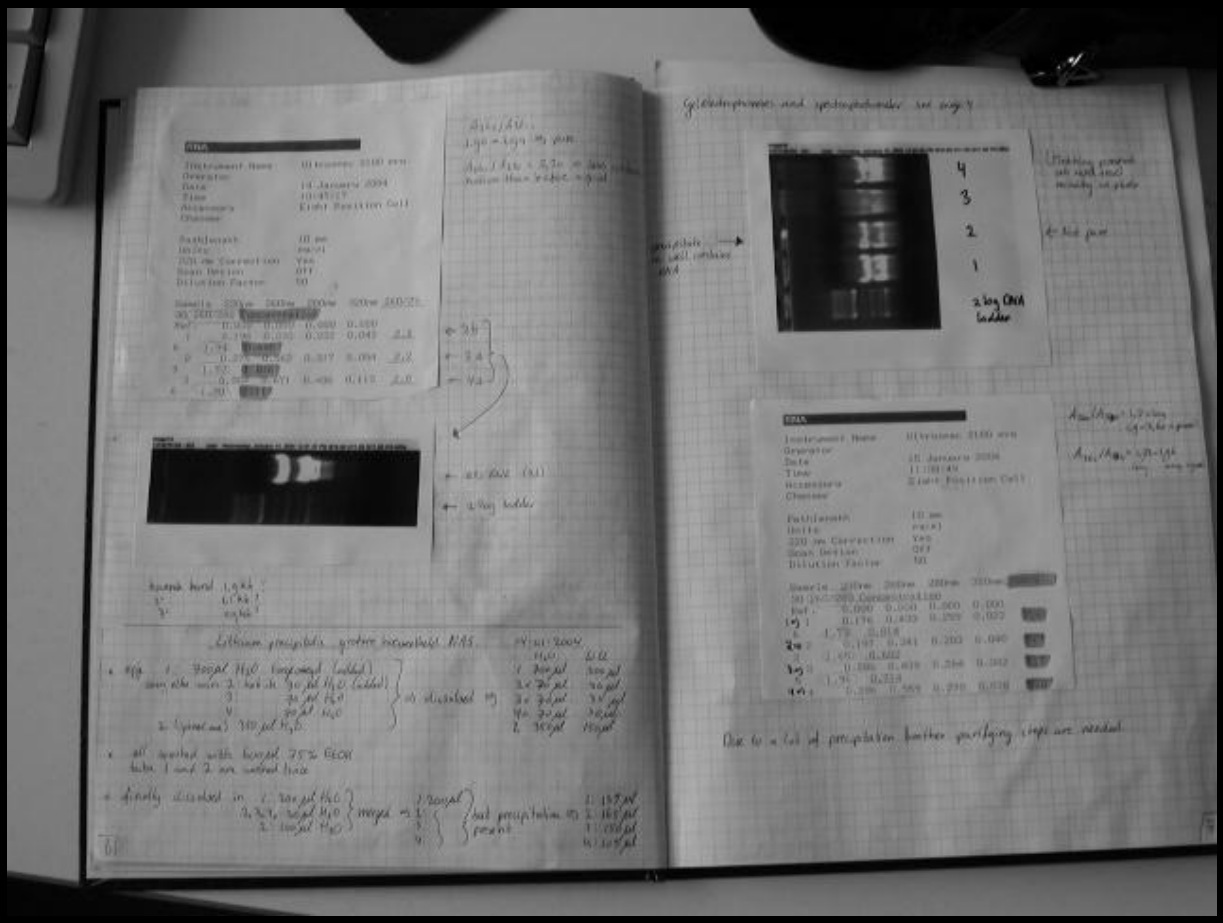
CHAPTER 7

Quest for the gene – as far as we got



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Introduction

A glucosinolate polymorphism, present in Dutch natural populations of *Barbarea vulgaris,* consists of two chemotypes. Plants of one chemotype contain mainly 2-phenylethyl-glucosinolate (gluconasturtiin, NAS-type) whereas plants of the other chemotype contain the hydroxylated form, (*S*)-2-hydroxy-2-phenylethyl-glucosinolate (glucobarbarin, BAR-type) (Van Leur et al., 2006). Our goal was to unravel the molecular mechanism behind this polymorphism. In the present chapter, we hypothesise about the class the gene belongs to and describe our efforts to identify this gene.

A qualitative polymorphism

Controlled cross-pollinations (Chapter 2, Van Leur et al., 2006) revealed that the chemotype is heritable and determined by a single gene with two alleles. The BAR-type allele proved to be dominant over the NAS-type allele. As the difference between the main glucosinolates in the two chemotypes is only one hydroxyl-group, it is most likely that the gene underlying this polymorphism is a hydroxylase.

The very low level of glucobarbarin detected in NAS-type plants indicates that the hydroxylase from BAR-type plants has a strongly reduced activity in NAS-type plants. There are many possible molecular mechanisms which may lead to reduced activity of the hydroxylase. First, the hydroxylase in NAS-type plants may be mutated in the open reading frame. This may yield a modified protein which still has some activity, but less than the intact hydroxylase in BAR-type plants. Alternatively, the enzyme activity can be reduced by regulation of the expression at any of the following levels: (1) by chemical and structural modification of DNA or chromatin, (2) transcription, (3) translation,(4) post-transcriptional modification, (5) RNA transport, (6) mRNA degradation or (7) post-translational modifications. Although the reduced hydroxylation in NAS-type plants can be caused by other enzymes or trans-acting factors, we started unravelling the molecular mechanism causing the polymorphism by identifying the hydroxylase.

Secondary modifications of glucosinolates

Genetic studies within *Brassica* and *Arabidopsis* indicate that side chain modification of aliphatic glucosinolates is regulated on three loci in the genome. Kliebenstein et al. (2001c) proposed a modular system that results in a high variety of glucosinolate products (Figure 7.1). The first step is an oxidation by *GS-OX*. The second step is a removal of the methylthio-group and the creation of a double bond by *GS-ALK* (Giamoustaris and Mithen, 1996; Li and Quiros, 2003). The third step is a desaturation and hydroxylation by *GS-OH* (Mithen et al., 1995).

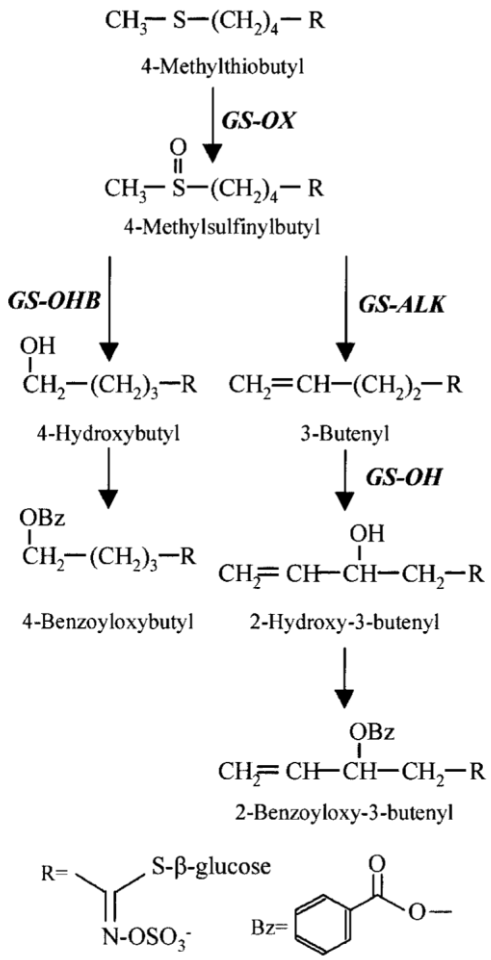

Figure 7.1 Side chain modifications of aliphatic glucosinolates in *Arabidopsis* (Kliebenstein et al., 2001b). Potential side chain modifications for the elongated methionine derivative C_4 homomethionine are shown. Steps with natural variation in *Arabidopsis* are shown in bold to the right or left of each enzymatic arrow with the name of the corresponding locus.

The reactions catalysed by the *GS-OH* and *GS-ALK* enzymes, utilise similar substrates and may have common reaction mechanisms. The gene corresponding to the *GS-OH* enzyme was identified as AOP3, and that of *GS-ALK* as AOP2. AOP2 and AOP3 genes originated from a gene duplication and both have a high homology to the 2-oxoacid-dependent dioxygenase enzymes (2-ODDs) (Kliebenstein et al., 2001b). When investigating the tissue specificity of the *GS-OH* enzymes in *Arabidopsis thaliana*, Kliebenstein et al. (2001c) detected three other *GS-OH* alleles that were responsible for the hydroxylation of 3-butenylglucosinolate into 2-hydroxy-3-butenylglucosinolate. The three alleles are located on a single locus, and fine scale-mapping identified a candidate 2-ODD gene (gene At2g25450, protein AAD20704.1) (D.J. Kliebenstein, unpublished data). Kliebenstein et al. confirmed the function of this candidate gene by: (a) the presence of a naturally occurring knock-out mutation of this gene which exhibits absence of the 2-hydroxy-3-butenyl glucosinolate, (b) tissue specific mRNA expression correlated closely to the hydroxylation activity, and (c) T-DNA knockout led to a complete loss of 2-hydroxy-3-butenyl glucosinolate production (unpublished data).

Based on the above information, we developed our working hypothesis that the conversion of 2-phenylethyl-glucosinolate into (*S)*-2-OH-2-phenylethyl-glucosinolate in *Barbarea vulgaris* BAR-type plants is performed by a 2-ODD with a great homology to above mentioned *GS-OH* responsible for the hydroxylation of 3-butenyl-glucosinolate. We expect that this gene has a lower activity in NAS-type plants, most likely because it has a mutation in the open-reading frame or in the promoter.

2-Oxoacid dependent dioxygenases

2-Oxoacid dependend dioxygenases (2-ODDs) are non-heme-iron-containing dioxygenases that typically use molecular oxygen, 2-oxoglutarate, ascorbate, and ferrous ions to catalyze substrate hydroxylations and other oxidations (Decarolis and Deluca, 1994). The gene family of 2-ODDs consists of cytoplasmic enzymes of which *A. thaliana* contains about 100 members (Pichersky and Gang, 2000; Prescott, 2000). They are involved in different biosynthetic processes such as biosynthesis of flavonoids, gibberellins (Hedden, 1997; Lange et al., 1997), ethylene, hydroxylation of proline residues, mugineic acid, auxin induced adventitious root formation (Butler and Gallagher, 1999) and also in biosynthesis of glucosinolates (Decarolis and Deluca, 1994; Hall et al., 2001; Kliebenstein et al., 2001b). Still, the function of many 2-ODDs is unknown (Prescott and Lloyd, 2000). The crystal structure is determined of the following 2-ODDs: an isopenicillin *N* synthase (IPNS) from the fungus *Aspergillus nidulans* (Roach et al., 1995), an anthocyanidin synthase (ANS) from *Arabidopsis thaliana* (Turnbull et al., 2001), and a 1-aminocyclopropane-1-carboxylic acid oxidase (ACCO) from *Petunia hybrida* (Zhang et al., 2004).

The 2-ODDs contain a common structural motif consisting of a 2-His-1-carboxylate facial triad, (active site: His-x-Asp + His). Their proteins have a molecular weight of 26 – 56 kD and the protein consists of alpha and beta subunits (Prescott, 2000; Clifton et al., 2006).

Interspecies approach: degenerate PCR

Known genes that affect similar processes in one organism could have their homologues amplified in other related organisms by the use of degenerate primer pairs. The use of degenerate Polymerase Chain Reaction (PCR) has proven to be a very powerful tool to find “new” genes or gene families. Homologous genes display regions where they are highly conserved and also regions where they are divergent, due to evolutionary processes. By aligning the protein sequences from a number of related proteins, one can identify shared conserved sequences which can be used as a starting point to make degenerate PCR primers. Degenerate primers have a number of options at several positions in the sequence to allow annealing to and amplification of a variety of related sequences. The degeneracy is computed by multiplying the level of degeneracy of each of the contributing amino acids. Here we perform degenerate PCR, using primers based on the protein sequence of AAD20704 (Kliebenstein *GS-OH*) and conserved 2-ODD amino acid sequences in order to identify candidates for the putative 2-ODD enzyme performing the conversion of gluconasturtiin to glucobarbarin.

Materials and Methods

Biological material

Seeds from the natural *B. vulgaris* population in Heteren the Netherlands (HE, 51.95 °N; 5.77 °E) were grown and the resulting BAR-type plants were selfed. To obtain homozygous BAR-type material, we selected three plants of a selfing that resulted in 100% BAR-type plants. Young leaves were chosen for extraction because they have high levels of glucobarbarin and are therefore expected to have a relatively high expression of the hydroxylation enzyme. Two grams of young leaf material was harvested per plant and immediately frozen using liquid nitrogen.

Primer design

As we wanted the primers to contain sequences of regions that are conserved in 2-ODD genes, we compared the *GS-OH* protein sequence (AAD20704.1) with a variety of other 2-ODDs. The amino acid sequences of similar or homologous proteins were retrieved by tBLASTN with the *GS-OH* nucleotide sequence (gene At2g25450) from the GenBank database (2004). The 100 sequences with the highest score were aligned using MegAlign 5.08 © (1993-2004 DNASTAR Inc). Based on clustering of the aligned sequences (ClustalW), we selected for every represented gene, the protein with the highest match, and made a new alignment with these selected proteins to identify the conserved regions.

Next to the alignment, we identified conserved regions by using BlockSimilarity scores, which is a measure of the length of a conserved sequence. A position in an alignment is conserved when the amino acid occupied by that position in all the sequences are either identical or “similar”.

To design our primers, we first selected conserved regions using BLOCKS (Henikoff and Henikoff, 1994) and obtained primer suggestions using CODEHOP (Rose et al., 1998). After manual adaptations to optimise the primers, we had 3 forward and 2 reverse primers (Table 7.1). The R2 primer was based on the conserved oxoglutarate binding site (Prescott and John, 1996).

Table 7.1 Sequence of the degenerate Forward (F) and Reverse (R) primers and their degeneracy.

primer		degeneracy
F2	GGNGTIAARGGNYTIGTNGA	
deg.	4 2 4 2 4	256
F3	GGITTYTTYCARGYIATHAAICAYGG	
deg.	2 2 2 2 3 2	96
F4	TTYGARHTIYTIWSIGARGC	
deg.	2 2 2 2 2 2	128
R2	GGYTRIGGRCAIGGNGGRSARYA	
deg.	2 2 2 4 2 2 2 2	512
R4	ARYTCYTTDATIGGICCRTA	
deg.	2 2 2 3 2	48

The degeneracy of the selected primers was kept as low as possible by including amino acids that have a low number of triplet codon possibilities (e.g. avoiding six fold sites of L, S, R and maximising one or two fold sites in the region). Another way by which we decreased degeneracy was by substituting four base wobbles with inosines, which will indiscriminately pair with adenine, thymine, or cytosine, thereby reducing degeneracy 4-fold.

To increase PCR efficiency, we added tails to the degenerate primers at the 5' ends to increase the primer length and hence annealing temperature. Although these tails do not have a function in the first few rounds, when the original cDNA template is amplified, the tails do match in subsequent PCR cycles when the PCR products containing the primers at each end are amplified. We used tails that have restriction sites that can be used for directional cloning. Additionally, they have terminal G's which encourages Taq polymerase to add overhanging A's for use in TA cloning. The tail of the forward primer (including EcorI site) was GCGCGGAATT and the tail of the reverse primer (including Hind3 site) was GCGCGCAAGCTT.

RNA isolation and cDNA synthesis

We used *B. vulgaris* cDNA because cDNA has lower complexity than genomic DNA and absence of introns results in a predictable fragment size. As commonly used Trizol-based plant RNA extraction methods yielded extremely low amounts of clean RNA, we isolated RNA using a modified RNeasy Maxikit for animal cells protocol (Qiagen, Crawley, W Sussex, UK). The protocol was modified by Raymond Hulzink (unpublished) in the following way: after the freezing, grinding and homogenization steps, all material was thoroughly mixed and divided over 4 Maxi-columns. The flow-through of these maxi columns was applied to second series of new RNeasy Maxi columns to enhance RNA yield. Subsequently, cDNA was synthesised using the iScript^{tm} cDNA synthesis Kit (BIO-RAD).

PCR

Degenerate PCR reactions were performed using 130ng of cDNA with 0.2 mM dNTPs, 100 pmol of each primer and 2 U of Taq polymerase (Expand High Fidelity PCR system, Roche Diagnostics, cat no: 1 759 078), with supplied buffer in a 20 µl reaction volume. The cycling conditions were 94 °C for 3 min, followed by 40 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min and a final extension at 72 °C for 5 min for the following primer combinations: F2-R4, F3-R2, and F4-R4 (Table 7.1).

Cloning & sequencing

PCR products were purified using QIAquick PCR purification kit protocol (Qiagen) and ligated in pGEM-T vector (Promega) and transformed into JM109 *E. coli* cells. Insert length was tested by colony PCR using the SP6 (5'-TAT TTA GGT GAC ACT ATA g-3') forward primer and T7short (5'-TAA TAC GAC TCA CTA TAG GG-3') reverse primers. The PCR reaction contained 0.2 mM dNTPs, 0.6µM of each primer and 0.06 U of Taq polymerase (Expand High Fidelity PCR

system, Roche Diagnostics, cat no: 1 759 078) with supplied buffer in a 25 µl reaction volume.

We selected 64 *B. vulgaris* inserts and sent them to Greenomics^{tm} (Plant Research International, Wageningen, The Netherlands) for sequencing. Manual editing of the sequence, contig assembly and ClustalW analysis was performed in BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). BLASTX analysis was done on the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/).

Results

Contig assembly analysis of the 64 *B. vulgaris* sequences resulted in 18 different contigs. A BLASTX analysis was performed to identify the most plausible frame for translation and the obtained results indicated that the translation products of six of these contigs showed a very high homology with different 2-ODD from *A. thaliana* (Table 7.2). For contig 2, the BLASTX alignment suggested that the sequence was derived from a gene with a deletion in the open reading frame causing a frame shift. A multiple alignment (ClustalW) of these six contig translation products with the 2-ODD from *A. thaliana* AAD20704, demonstrated an overall high sequence homology (Figure 7.2).

Table 7.2 BLASTX alignment results of the contigs of *B. vulgaris* PCR products and their percentage identity and and similarity with AAD20704.

	BLASTX highest homology	**coding gene**	**Expect value**	**% identity**	**% similarity**
Contig 1	putative 2-ODD (*A. thaliana*)	AT2G25450	3.00E-94	73	85
Contig 2	putative 2-ODD (*A. thaliana*)	AT2G25450	1.00E-86	61 (68)	75 (79)
Contig 3+10	putative 2-ODD (*A. thaliana*)	AT2G30840	2.00E-115	89	95
Contig 4	putative 2-ODD (*A. thaliana*)	AT1G06650	2.00E-62	93	95
Contig 5+11	putative 2-ODD (*A. thaliana*)	AT1G06640	3.00E-115	89	93
Contig 6	putative 2-ODD (*A. thaliana*)	AT1G06620	8.00E-47	85	93

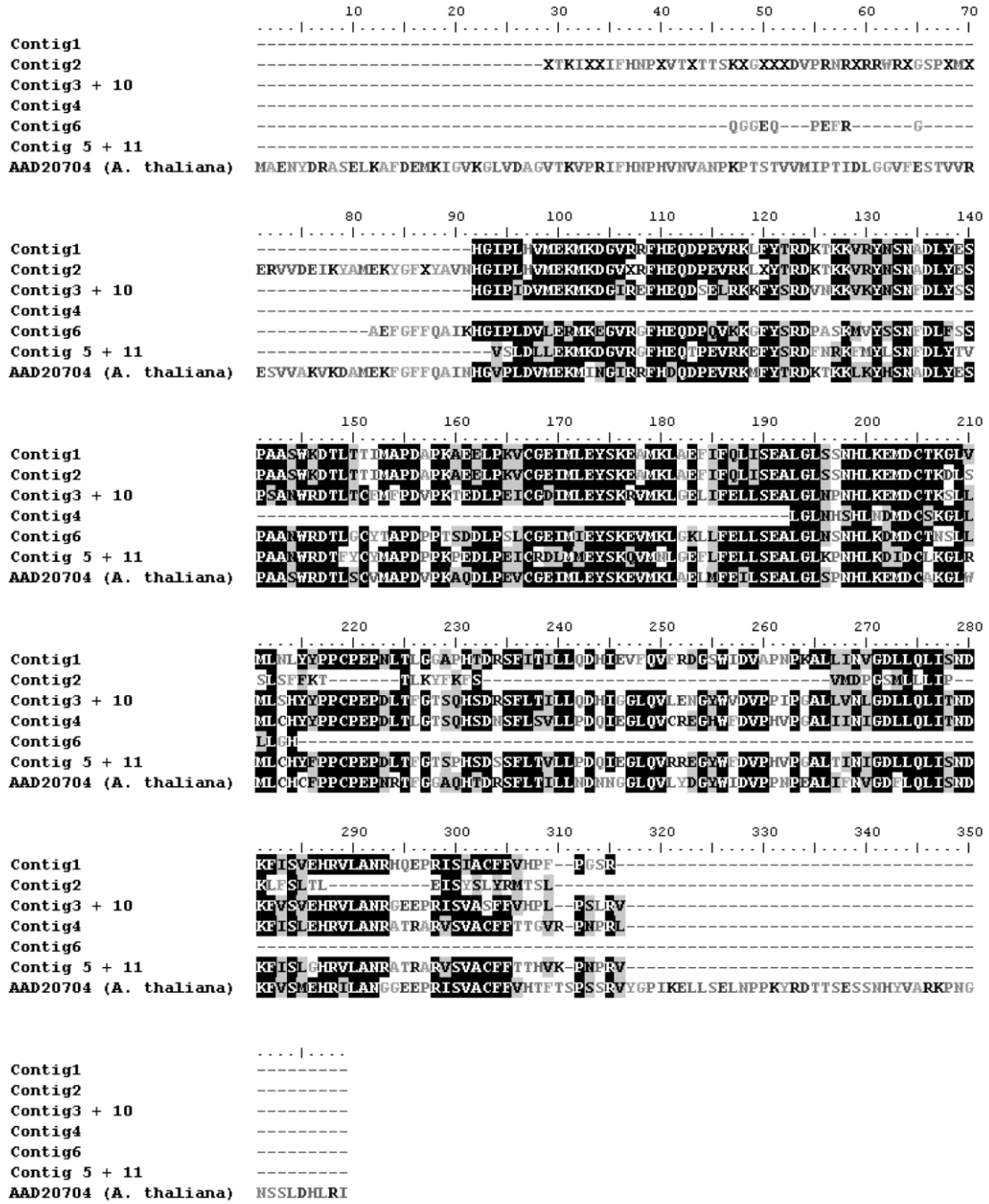

Figure 7.2 Multiple alignment (ClustalW) of the six *B. vulgaris* contig translation products with the 2-ODD from *A. thaliana* AAD20704. Identical amino acids are in black and similar amino acids in grey background.

Discussion & Future directions

In order to identify the gene responsible for the BAR/NAS polymorphism in *B. vulgaris,* we used an interspecific approach. This approach was successful in that we amplified *B. vulgaris* sequences with a high similarity to *A. thaliana* genes coding for enzymes with similar functions. Contig 1 and 2 had the highest similarity to the hydroxylase responsible for the hydroxylation of 3-butenyl-glucosinolate in *A. thaliana.* Hence, these sequences are currently the most likely candidates for the hydroxylation of 2-phenylethyl-glucosinolate in *B. vulgaris.* Further characterisation of these candidate genes is required to see whether they are indeed involved in the *B. vulgaris* polymorphism. A first step to take would be the comparison of the expression levels of the candidate genes between BAR-type and NAS-type plants e.g., by quantitative RT-PCR. If the expression differs between the chemotypes, the enzyme is likely to be involved in causing the different chemotypes.

A second approach is to retrieve the genomic sequence from the candidate genes in both BAR- and NAS-type plants. This enables comparison of the gene sequence (open reading frames as well as promoters) between both chemotype plants, to detect possible mutations in the NAS-type sequence. The functionality of the gene and its resulting enzyme should than be confirmed by, e.g. heterologous expression, enzyme essays, and the use of knock-out mutants. Further characterisation of the identified candidate hydroxylases here may reveal the mechanism of this polymorphism, and ultimately, be applied to study the molecular-evolutionary aspects of this defence polymorphism in natural populations.

Acknowledgements

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CHAPTER 8

Discussion and Synthesis

Hanneke van Leur

Barbarea vulgaris is polymorphic for glucosinolates

Glucosinolates are a highly diverse group of defence compounds, with over 120 members (Fahey et al., 2001). When I sampled leaves from *Barbarea vulgaris* plants in natural populations from the Netherlands and surrounding countries, I detected the following six glucosinolates: glucobarbarin, gluconasturtiin, glucosibarin, glucobrassicin, 4-methoxyglucobrassicin and neoglucobarbarin (Chapter 2). In most *B. vulgaris* plants, glucobarbarin comprised 94% of the total glucosinolate content. These plants are, therefore, named BAR-type plants. In eight natural populations I detected that 2-22% of the plants contained the same glucosinolates as above, but in different proportions. Plants with this 'new' *B. vulgaris* glucosinolate profile contained 82% gluconasturtiin, and they are therefore called NAS-type plants (Chapter 2).

The main aim of the research presented in this thesis was to identify physiological and ecological processes that may explain the existence of this defence polymorphism. In this thesis I studied the molecular-genetic mechanism behind this polymorphism and compared the costs and benefits associated with the two chemotypes. The quantified metabolites, plant fitness characteristics and the effect on invertebrate herbivores are compared between BAR-type and NAS-type plants (Figure 8.1).

The chemotype is heritable

Glucosinolate-targeted HPLC analyses of rosette leaves, stem leaves, roots, flower and seeds showed that in all these tissues BAR-type plants had a high glucobarbarin content and NAS-type plants a higher level of gluconasturtiin. Thus, the chemotype is consistently expressed in all tissues. When I induced plants by application of jasmonic acid to roots or shoots (Chapter 2), with shoot feeding *Pieris rapae* or *Mamestra brassicae* larvae (Chapter 3), or with root feeding *Delia radicum* larvae (Chapter 4), the chemotype did not change. This underscores the consistency of the BAR- or NAS-chemotype. These observations suggested that the chemotype was fixed in the genotype of the plant. Several rounds of controlled crosses confirmed that the chemotype was genetically determined by a single gene with two alleles. The allele coding for the BAR-type was dominant and the allele for the NAS-type was recessive (Chapter 2).

Glucosinolates differ more than other metabolites

The genotypically deterimed difference in glucosinolate content may have pleiotropic effects that cause other metabolites to differ too. Therefore, I analysed the metabolomes of the chemotypes using an untargeted LC-QTOF-MS approach. I was not interested in epigenetic effects that are unique for a specific genetic background. Therefore, I included plants from two different half-sib families and focussed on differences between the chemotypes that were consistent in both families. Similar to the targeted HPLC analyses (Chapter 2), the untargeted metabolomic analysis revealed that glucosinolates (mostly glucobarbarin/gluconasturtiin) and glucosinolate-related compounds differed with high significance between the chemotypes. Apart from glucosinolates, only 11 thus far

unidentified compounds were significantly different between the chemotypes, five of which were identified in shoot tissue, and six in root tissue (Chapter 3).

In addition to the untargeted analyses, the LC-QTOF-MS data showed that the levels of saponins, flavonoids and some common plant acids (chlorogenic- , citric-, coumaric, and ferulic acid) were not different between the chemotypes. Even the saponins that were responsible for differential herbivore performance in a Danish *B. vulgaris* polymorphism (Shinoda et al., 2002; Agerbirk et al., 2003b), did not differ between BAR-type and NAS-type plants.

Although the LC-QTOF-MS analyses detected many metabolites in shoots and roots, nutritionally important primary metabolites such as amino acids and sugars were hardly detected. Therefore, I studied the content of water-soluble sugars and amino acids between the chemotypes using targeted HPLC analyses. Non-induced plants (Chapter 4), plants induced by shoot feeding *Mamestra brassicae* or *Pieris rapae* larvae (Chapter 3), and plants induced by root feeding *Delia radicum* larvae (Chapter 4), showed no difference between the chemotypes neither in total sugar or amino acid levels nor in the levels of individual sugars or amino acids.

Chemotypes do not differ in direct costs

The major qualitative differences in glucosinolate content and minor differences in correlated metabolites may be accompanied by differential direct costs. Allocation of resources to defence is generally considered to be costly when it, in the absence of herbivory, negatively affects survival, growth or reproduction of the plants and thus plant fitness (Simms and Rausher, 1987; Simms and Fritz, 1992; Karban and Baldwin, 1997; Strauss et al., 2002). Seeds with a NAS-type glucosinolate profile had a higher seed weight than BAR-type seeds, but this difference in seed weight never translated into different germination rates or germination success, different seedling size, different production of biomass, number of leaves, different leaf morphology or different number of flower stalks (Chapter 3). Based on these result, I conclude that the plants were not nutrient-limited or stressed by competition. The chemotypes do not differ in direct costs.

Chemotypes accrue different ecological costs

Under controlled conditions

Although the glucosinolates only differ in one hydroxyl-group (glucobarbarin is hydroxylated, gluconasturtiin is not), the bioactive breakdown products are expected to be very different. NAS-type plants most likely produce mainly isothiocyanates and BAR-type plants oxazolidine-2-thiones. Instead of quantifying these breakdown-products, I focused on plant-herbivore interactions. In general, the broad toxicity of isothiocyanates suggests that NAS-type plants will be highly resistant. However, in experiments under controlled conditions, the larvae of the generalist moth *Mamestra brassicae* preferred to feed from NAS-type leaves instead of BAR-type leaves. When the larvae were forced to stay on BAR-type plants, they hardly ate, hardly gained weight and died quickly (Chapter 4). Remarkably, adult *M.*

brassicae moths did not discriminate between the chemotypes for their oviposition. In this case, the 'Mother knows-best' hypothesis did not fit the observations (Jaenike, 1978; Johnson et al., 2006).

This radical 'live-or-death' difference in herbivore performance between the chemotypes was not shown for any other herbivore in this study. However, a difference in performance between the chemotypes was found for the root feeding *Delia radicum* (Chapter 5). Larvae of *D. radicum* developed pupae with a higher mass when feeding on BAR-type plants compared to NAS-type plants. Also for oviposition, *D. radicum* tended to prefer the BAR-type. For the shoot feeding specialist butterfly *Pieris rapae*, there were no differences in preference of performance between the chemotypes (Chapter 4).

Under semi-field conditions

To include the complexity and dynamics of ecological interactions in natural populations, I planted both chemotypes in a mixed pattern in a common garden. Two-years of weekly assessments of the aboveground invertebrate community revealed that flea beetles (*Phyllotreta sp)* and the gall midge *Contarinia nasturtii* were more abundant on BAR-type plants, whereas *Pieris rapae* preferred NAS-type plants. The sap-sucking species that were observed, *i.e.* froghoppers (*Cercopoidea)*, the specialist aphid *Brevicoryne brassicae* and the generalist aphid *Myzus persicae* did not discriminate between the chemotypes (Chapter 6). Belowground, *Delia radicum* pupae tended to be more abundant on BAR-type plants. Neither ecto- or endoparasitic plant feeding, fungi-, bacteria feeding or omni-carnivorous nematodes nor the nematode community composition differed between the chemotypes (Chapter 6). Despite some clear differences in aboveground herbivory, the BAR-type and NAS-type plants did not show fitness differences (measured as number of seed capsules, root and shoot biomass development and rosette diameter) (Chapter 6). So, some herbivore species have a different abundance on BAR-type and NAS-type plants, but this did not translate into fitness difference.

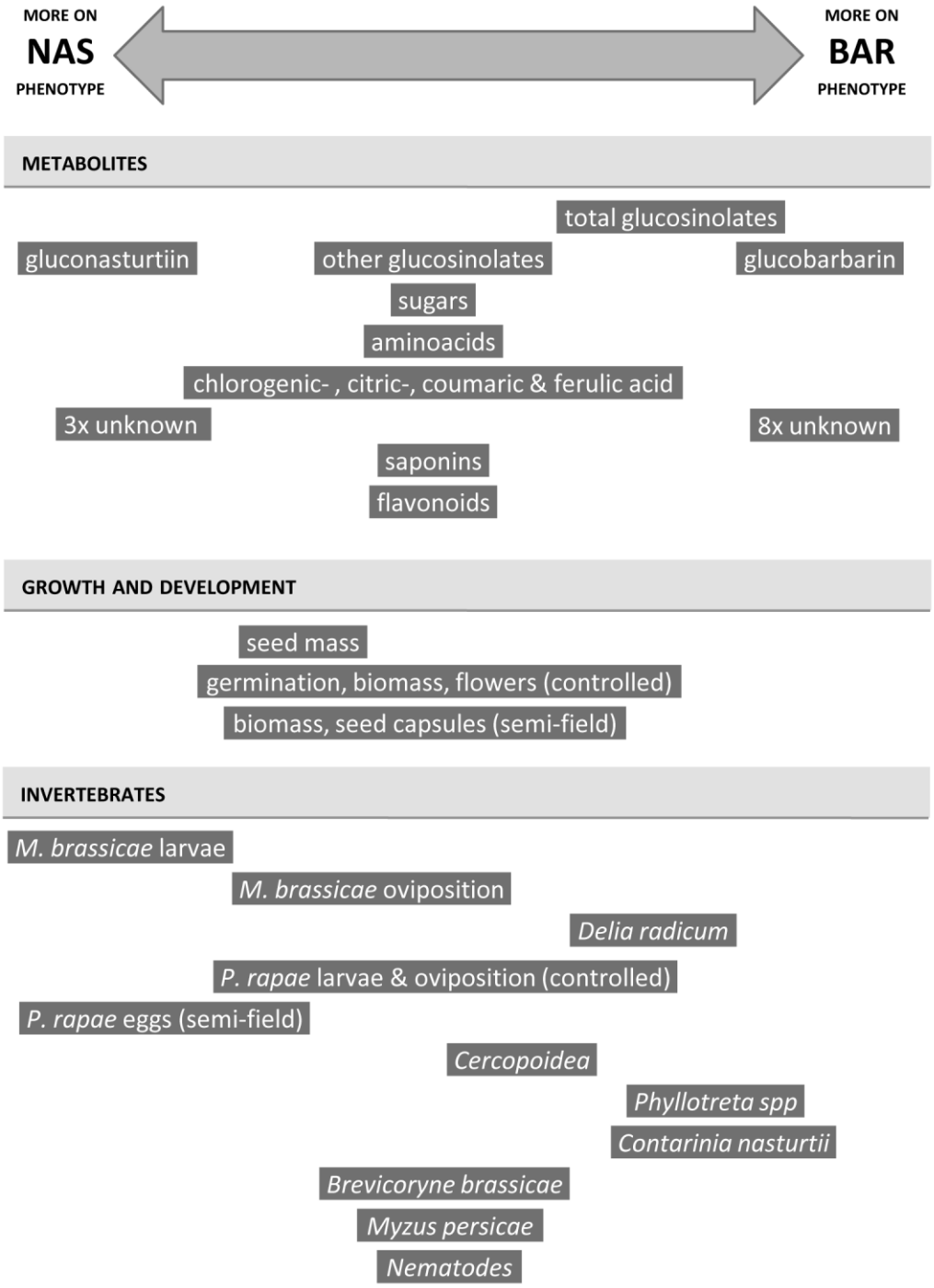

Figure 8.1 Comparison of BAR and NAS-type plant metabolite content, growth and development and invertebrates associated. A bar more to the right indicates that the trait was more prominent in or the herbivores were more abundant on BAR-type plants, more to the left means more on NAS-type. When positioned in the centre there was no difference between the chemotypes. The length of the bar is given by the length of the text and is not an indicator of the importance of the effect.

Root versus shoot chemistry

Plants are attacked on aboveground tissues, but also belowground herbivory can have a strong impact on plant performance and plant biodiversity (Müller-Schärer and Brown, 1995; Van der Putten et al., 2001; Wardle, 2002; De Deyn et al., 2003; Poveda et al., 2005). Detailed reviews have highlighted the effects of root-feeding insects (Andersen, 1987; Brown and Gange, 1990) and root-feeding nematodes (Stanton, 1988; Mortimer et al., 1999). Therefore, plants also defend themselves against belowground attackers. Whereas structural plant defences, such as spinescence, pubescence, sclerophylly and raphides, are mainly found in shoot tissues, many chemical defence compounds, such as terpenoids and pyrrolizidine alkaloids are also present in roots (Van Dam et al., 1995; Walker et al., 2003; Wuyts et al., 2006). Glucosinolates are also found in all tissues, but their composition and abundance is often tissue specific (Sang et al., 1984; Brown et al., 2003; Halkier and Gershenzon, 2006).

In the *B. vulgaris* glucosinolate chemotypes, the prevalence of the main glucosinolate in the profiles is consistent in aboveground and belowground tissues. However, the glucosinolate patterns in shoots, seeds and flowers were much more distinctly different between the chemotypes than in roots (Chapter 1). The less pronounced distinction between the roots of the chemotypes is confirmed for rosette plants in the HPLC analyses in chapter 4 and 5, and in the LC-QTOF-MS analyses in chapter 3. All analyses show that, consistent with the shoot patterns, glucobarbarin levels were always much higher in BAR-type roots. Gluconasturtiin levels, on the other hand, were much higher in NAS-type shoots, but were not or only slightly higher in NAS-type roots. The absence of a difference in root gluconasturtiin levels between the chemotypes is not caused by low gluconasturtiin levels in NAS-type roots, but is rather due to high levels of gluconasturtiin in BAR-type roots (Chapter 1). This relatively high gluconasturtiin content in root tissue is typically found in various crucifer species, whose shoots hardly contain gluconasturtiin (Sang et al., 1984; Kirkegaard and Sarwar, 1998; Agerbirk et al., 2003a; Van Dam et al., 2004). Moreover, the untargeted metabolomics comparison between the chemotypes (Chapter 3), showed that more mass signals differed between shoots than between roots and that the root mass signals differed with a lower statistical significance than the shoot mass signals.

Another difference in glucosinolate-mediated defences is shown between roots and shoots of *A. thaliana.* Burow et. al. (2007) demonstrated that the epithiospecifier protein, which directs glucosinolate hydrolysis towards nitrile formation, was present in all aboveground organs, but not in roots. Also, the hydrolysis enzyme, myrosinase mainly accumulates in the roots of mature *B. napus* plants (Bones, 1990). This would mean that, even when glucosinolate content is identical in roots and shoots, they can be differentially defended because they form different sorts or different amounts of glucosinolate breakdown products.

Aboveground versus belowground chemotype-herbivore interactions

The more distinct differences of the chemotypes aboveground, compared to belowground, translated into more clear differential effects of the chemotypes on shoot herbivores than on root herbivores. The most dramatic effect on herbivore performance and preference was shown for the shoot-feeding *Mamestra brassicae,* where the difference between BAR-type and NAS-type plants meant the difference between life and death (Chapter 4). Additionally, the shoot feeding *Pieris rapae, Phyllotreta spp,* and *Contarinia nasturtii* also showed differences between BAR-type and NAS-type plants, when these plants were placed in equal frequencies in a common garden (Chapter 6). In contrast, root herbivores hardly discriminated between the chemotypes. The root feeding *Delia radicum* only showed a small difference in pupal weight between the two types, and plant parasitic nematodes showed no differences at all. Bacteria feeding, fungal feeding and omni-carnivorous nematodes in the rhizosphere did not show differences in number or composition between the chemotypes either.

Natural variation: chemotypes vs. family

Most experiments were performed using half-sibs. As half-sibs have a high genetic overlap for maternal genes, the epistatic effects can be highly similar. So, looking within one half-sib family reduces the overall variation. This enabled me to elucidate subtle chemotype effects. On the other hand analyses within only one half-sib family, increases the chances of studying exceptional phenotype, which does not represent the natural population increases. As I was only interested in chemotype effects that not depend on a certain genetic background, I included at least two half-sib families in my studies. This increased the overall variation in all datasets.

For metabolites as well as for seed mass, seedling characteristics, rosette diameter and number of flowering plants we found significant variation between half-sib families. Although this complicated the analyses and the interpretation of the data, the use of more than one half-sib families enabled me to compare variation between chemotypes and half-sib families.

For example, the metabolomics multivariate analyses in chapter 3, showed that metabolites varied between chemotypes when both half-sib families were taken together, but a separate analysis of each half-sib family revealed even stronger chemotype effects. A comparison of the chemotype effects between the half-sib families enabled discrimination between metabolites that only varied in a specific background and those that consistently varied between chemotypes. I also used the half-sib families for the interpretation of the amino acid and sugar levels (Chapter 4). Both groups of primary metabolites showed a chemotype effect within a half-sib family, but in one family they were higher in BAR-type plants and in the other in NAS-type plants. As the effects on *Mamestra brassicae* performance were similar on both families, I could exclude the differences in primary metabolites as explaining factor. If I had only used one half-sib family, I would not have been able to discriminate between nutrient and chemotype effects.

Maintenance of a polymorphism

By sampling natural populations in The Netherlands and surrounding countries, I found that the chemotypes co-occurred in several populations (Chapter 2). If the populations were to meet the assumptions of a Hardy-Weinberg Equilibrium the recessive phenotype, the NAS-type, is expected to comprise 25% of the individuals within a population (Falconer, 1981). As the sampled natural populations of *B. vulgaris* only consisted of 0-22% NAS-type plants, the populations did not meet all assumptions. There are several potential options for this discrepancy from a balanced Hardy-Weinberg Equilibrium, which I will discuss below.

First, it may be possible that the chemotypes do not cross randomly, but assortative. This may happen when individual pollinators prefer one chemotype over the other. Second, *B. vulgaris* grows in dense vegetations in which seedling establishment may be less important for establishment than clonal reproduction. Alternatively, the polymorphism may not be in a stable state yet or anymore. For example, the NAS-type may be relatively new and the polymorphism has not yet reached a stable state, or the populations were in a stable state, but the conditions have changed in a way that the NAS-type had a selective disadvantage. The last option is that the chemotypes have an alternative equilibrium due to frequency dependent selection (Maynard Smith, 1989), in which the fitness of the BAR phenotype is dependent on the relative frequency of the NAS phenotype. The results of the experiments described in this thesis, do not provide sufficient information to reject or support any of the above possibilities.

***Barbarea vulgaris* and its glucosinolates as a model**

The most widely used plant model species is *Arabidopsis thaliana*. It is very suitable for studying wide-ranging topics including plant development, genetics and pathogen resistance (Kliebenstein, 2004). However, in spite of its obvious benefits, *A. thaliana* also has limitations for ecological studies, because it has a very specific defence strategy (Harvey et al., 2007). It is a small 'fugitive' species, and early season growth may lead to a phenological mismatch between *A. thaliana* and the vast majority of its potential herbivores (Pigliucci, 2002; Arany et al., 2005; Hoffmann, 2005). Therefore, including other life history strategies can be helpful in getting a more complete picture of plant defence strategies. *Barbarea vulgaris* is a perennial, and exposed to a much wider range of ecological interactions with phytophages and competitors (Hegi, 1962). The experimental garden experiment (Chapter 6) confirmed that *B. vulgaris* indeed has to deal with a wide range of species: shoots were attacked by several aboveground herbivore species and a diverse community of nematodes colonized the soil surrounding the roots.

Although the ecology of *B. vulgaris* and *A. thaliana* is very different, they may have similarities as well. Both belong to the Brassicaceae, contain glucosinolates and most likely show quite some genetic homologies (Al-Shehbaz et al., 2006; Bailey et al., 2006). This enabled me to use information of biosynthetic enzymes from *A. thaliana* in the search for the gene which catalyses the hydroxylation of *B. vulgaris* (Chapter 8). This approach was successful because it resulted in the identification of some candidate genes. However,

further research is still required to elucidate whether these candidate genes are differentially expressed in the two chemotypes.

Directions for future research

In this thesis I studied the *B. vulgaris* glucosinolate polymorphism on many functional levels: molecular, genetic, metabolite, metabolome, plant organ, plant interactions with a single herbivore and with an herbivore community. Still many interesting aspects remain unknown. In my opinion, the following aspects should get priority in future research.

1. Identification and quantification of the actual glucosinolate breakdown products: For example, by targeted isolation of isothiocyanates and oxazolidine-2-thiones and analyses of plant volatiles by GC-MS. As a follow-up, the bioactive compounds in these isolates could be identified using bio-assay guided fractionation.

2. Include higher trophic level interactions:

Glucosinolates and breakdown products do not only affect herbivores but also higher trophic level organisms (Soler et al., 2005; Soler et al., 2007a). Plant volatiles may direcly attract natural enemies of herbivores (Price et al., 1980; Barbosa and Saunders, 1985; Dicke, 1995; Godfray, 1995; Turlings and Benrey, 1998). Indirectly, the plant may affect herbivore quality which in turn changes the interactions with their predators, parasitoids and even hyperparasitoids (Müller et al., 2001; Harvey et al., 2003; Soler et al., 2007b). Therefore, it would be interesting to search for differences in host and host plant preference of specialist and generalist parasitoids and even higher trophic levels. Hereby I would expect that, due to the volatility of 2-phenylethyl isothiocyanate, the NAS-type plants would attract more (specialist) natural enemies, and thus would benefit more from top-down control of its herbivores.

3. Identification of the gene responsible for the B. vulgaris polymorphism:

Although the biosynthesis pathways of indole and aliphatic glucosinolates (Celenza, 2001; Mithen, 2001a; Mikkelsen et al., 2002), especially the chain elongation in of aliphatic glucosinolates (Graser et al., 2001; Falk et al., 2004; Textor et al., 2004; Tokuhisa et al., 2004) are heavily investigated, only little is known about the biosynthesis of aromatic glucosinolates (Halkier and Du, 1997; Wittstock and Halkier, 2000; Kliebenstein et al., 2001a). The identification of the gene causing the polymorphism can shed some light on the secondary modification of glucosinolates. If the hydroxylation is regulated on the level of gene expression, quantitative PCR techniques may reveal how B. vulgaris chemotypes establish different proportions of hydroxylated to non-hydroxylated glucosinolates in the roots compared to shoots.

4. Inter- and intraspecific plant competition:

Glucosinolates may play a direct role in interspecific plant competition, because glucosinolates in root exudates can suppress germination of competitive species, such as grasses (Siemens et al., 2002). For *B. vulgaris*, which frequently occurs in established grass- and herb communities (Hegi, 1962), interspecific competition is an important ecological

processes in the establishment of this species. On the other hand *B. vulgaris* may grow *en masse* as a pioneer species in recently disturbed soils. Therefore interspecific competitive ability can be crucial for survival. Therefore, next to plant-herbivore interactions, plant-plant competition of the two chemotypes should be investigated.

5. Up-scaling to comparisons between populations:

One of the triggers for studying costs and benefits of the *B. vulgaris* BAR/NAS chemotypes was the observation that the relative frequencies of the chemotypes within a population differed between populations The work presented in this thesis revealed that some herbivores discriminate between the chemotypes and suggests that the local composition of the herbivore community may determine the relative fitness of the chemotypes. Therefore, I propose to upscale the research to the population level by linking herbivore communities in natural populations to the BAR/NAS frequencies in those populations.

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Samenvatting

Net als veel andere planten, verdedigt Gewoon Barbarakruid (*Barbarea vulgaris*) zich met chemische afweerstoffen tegen belagers. Hiertoe produceert het Barbarakruid zogenaamde glucosinolaten. Deze stoffen komen voor in veel kruisbloemigen, zoals koolsoorten, mosterd en de wetenschappelijke modelplant Zandraket (*Arabidopsis thaliana)*. Glucosinolaten vormen samen met een enzym, myrosinase, een tweecomponentensysteem: het enzym en de glucosinolaten zijn gescheiden opgeslagen, maar als het blad beschadigd wordt, komen ze bij elkaar en worden de glucosinolaten door het enzym omgezet. Voor generalistische belagers zijn de glucosinolaten en de omzettingsprodukten vaak giftig, waardoor ze vraatschade aan planten tegengaan. Specialistische belagers kunnen deze stoffen daarentegen gebruiken als kenmerk voor een geschikte waardplant. Op deze aangepaste herbivoren hebben glucosinolaten juist een aantrekkende en vraatstimulerende werking. De omzettingsprodukten veroorzaken ook de smaak van de vele kool- en mosterdsoorten die voor menselijke consumptie worden gebruikt. Er zijn meer dan 100 verschillende glucosinolaten, die elk een verschillende chemische structuur hebben. Die structuur is bepalend voor het eindproduct, dat gevormd wordt bij het beschadigen van de plant. Elke plantensoort heeft zijn eigen typische samenstelling van glucosinolaten. Het doel van mijn onderzoek was te onderzoeken of de verschillen in glucosinolaatprofiel ook van invloed zijn op de resistentie tegen planteneters.

In een aantal Nederlandse populaties van Gewoon Barbarakruid is gevonden dat niet alle planten precies dezelfde glucosinolaten maken. Het grootste deel van de planten maakt voornamelijk glucobarbarine, een glucosinolaat dat typisch is voor deze plantensoort en naar het geslacht *Barbarea* vernoemd is. Ook in populaties in Duitsland, België, Frankrijk en Zwitserland vond ik alleen maar planten met voornamelijk glucobarbarine. In de helft van de Nederlandse populaties maakte een klein deel van de planten (2-22%) een andere glucosinolaat, gluconasturtiine genaamd. Het verschil in de structuur van deze twee glucosinolaten is zeer klein; glucobarbarine heeft slechts één hydroxygroep meer dan gluconasturtiine. Dit kan biologisch gezien echter zeer belangrijk zijn: als gluconasturtiine in contact komt met myrosinase wordt er een giftig en pittig smakend isothiocyanaat gevormd, terwijl in planten met glucobarbarine, door de positie van de hydroxygroep, zogenaamde oxazolidinethionen ontstaan. Van deze oxazolidinethionen is niet bekend of ze acuut giftig zijn, maar wel dat ze in zoogdieren de opname van jodium remmen, waardoor problemen met de schildklier kunnen ontstaan. Het *Barbarea vulgaris* glucosinolaatpolymorfisme bestaat dus uit twee chemotypen. In dit proefschrift karakteriseer ik de *B. vulgaris* chemotypen en gebruik ik dit polymorfisme om effecten van verschillende glucosinolaten op planteneters te bestuderen.

Het verschil in glucosinolaatprofiel is consistent aanwezig in alle organen van *B. vulgaris*, maar het verschil is groter in de bovengrondse plantendelen dan in de wortels. Het glucosinolaatprofiel verandert niet wanneer de planten door insecten of kunstmatig, door toevoeging van jasmonzuur, geïnduceerd worden. Met behulp van kruisingsproeven heb ik aangetoond dat het vermogen om glucobarbarine te produceren erfelijk is en gereguleerd wordt door een dominant gen. Op basis van de aanname dat er een specifiek enzym is dat de hydroxylering van gluconasturtiine tot glucobarbarine verzorgt, heb ik enkele

kandidaatgenen geïdentificeerd. Verder onderzoek is nodig om vast te stellen of één van deze kandidaatgenen daadwerkelijk de basis vormt voor het verschil tussen de chemotypen.

Vervolgens heb ik onderzocht welk effect het verschil in chemotype heeft op blad- en wortelherbivoren. De planten met glucobarbarine bleken zeer resistent tegen de generalistische bladetende rupsen van de mot *Mamestra brassicae*. Slechts enkele rupsen overleefden op planten met glucobarbarine en als ze konden kiezen, hadden ze een sterke voorkeur om te eten van planten met gluconasturtiine. De vrouwtjesmotten legden echter ongeveer evenveel eitjes op bladeren van beide chemotypen. Rupsen van het specialistische kleine koolwitje groeiden even goed op beide typen planten en maakten ook in de keuzetoets geen onderscheid. De larven van de specialistische koolwortelvlieg deden het echter slechter op wortels van het gluconasturtiine type dan op wortels van het glucobarbarine type. Ik heb ook gemeten wat het gevolg is voor de plant van een infectie met wortelvliegen. Hieruit bleek dat de massa van de wortels en de scheuten van beide chemotypen halveerde door de wortelvliegen en dat de gehaltes van voedingstoffen zoals suikers en aminozuren afnamen.

Om de resultaten uit de kas beter te kunnen vertalen naar de natuurlijke situatie, heb ik in een proeftuin planten van beide chemotypen geplant en gedurende twee jaar elke week van het groeiseizoen de aantallen herbivoren geteld. Het bleek dat een aantal bovengrondse insecten wel voorkeur vertoonden voor een bepaald chemotype en andere soorten niet. Vlinders van het kleine koolwitje legden bij voorkeur hun eieren op planten met gluconasturtiine, maar aardvlooien en galmuggen kwamen meer voor op planten met glucobarbarine. Koolluis en perzikluis hadden geen voorkeur en kwamen op beide chemotypen evenveel voor. Drie tot vier keer per jaar heb ik een aantal planten uitgegraven om de wortelherbivoren en de nematoden in de grond te analyseren. De aantallen en soortsamenstelling van deze ondergrondse organismen verschilden niet tussen de chemotypen.

Tenslotte heb ik uitgebreide metabolomische analyses uitgevoerd met behulp LC-TOF-MS om de chemische verschillen tussen de twee chemotypen nader te onderzoeken. Multivariate analyses toonden aan dat glucosinolaten inderdaad het grootste chemische verschil tussen de typen veroorzaken en dat deze verschillen groter zijn in de scheut dan in de wortel. Naast de glucosinolaten vonden we slechts acht niet nader geïdentificeerde stoffen die in kleine hoeveelheden voorkwamen en verschilden tussen de chemotypen. Bekende afweerstoffen zoals flavonoïden en saponines zijn wel geïdentificeerd, maar verschilden niet tussen de chemotypen. Daardoor is het zeer waarschijnlijk dat de verschillen in groei en voorkeur van herbivoren voornamelijk het gevolg zijn van de verschillen in glucosinolaatprofiel

Op basis van de kas- en veldproeven concludeer ik dat de structuur van de glucosinolaten significante verschillen veroorzaakt in resistentie tegen verschillende herbivoren. Het is echter niet zo dat het ene chemotype in alle gevallen meer resistent is dan het andere chemotype. Welk van de twee chemotypen een voordeel heeft, hangt dus af van welke herbivoren in een bepaalde plantenpopulatie voorkomen. Op deze manier kan het gevonden glucosinolaatpolymorfisme in natuurlijke populaties gehandhaafd blijven.

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MTI: The interacting department of Multitrophic Interactions (summer 2006)

The *Barbarea* fieldwork team (left to right): Freddy ten Hooven, Ciska Raaijmakers, Nicole van Dam, Hanneke van Leur and Gregor Disveld.

Curriculum Vitae

Hanneke van Leur was born on the 19th November 1978, in 's-Hertogenbosch, the Netherlands. She attended the Frederik Hendrik college in 's-Hertogenbosch for her secondary education (Atheneum). In 1997 she started her study biology at Radboud University in Nijmegen. As part of her masters she carried out several undergraduate research projects. In the first project, she studied the regulation of the development of constitutive aerenchyma, supervised by Eric Visser of the Department of Experimental Plant Ecology. The second research project was an EST analysis of the molecular regulation of female gametophyte development, performed at the Department of Plant Cell Biology and supervised by Koen Weterings. For a third project she travelled to the Bolivian amazone forests, to study the effects of habitat on cashing of Brazil nuts. This research was coordinated by Utrecht University in collaboration with Programa Manejo de Bosques de la Amazonia Boliviana (PROMAB) and supervised by Jacaranda van Rheenen and Pieter Zuidema. As a last project, she set-up a master course on microbial ecology, supervised by Marc Strous. She obtained her MSc degree in 2003.

In October 2003, she started her PhD in the Department of Multitrophic Interactions at the Centre for Terrestrial Ecology of the Netherlands Institute of Ecology (NIOO-KNAW). Under supervision of Nicole van Dam, Wim van der Putten and Louise Vet, she studied the genetics, chemistry and ecology of a qualitative glucosinolate polymorphism in *Barbarea vulgaris.* Part of the molecular work was performed at the Department of Biochemistry of the Max Planck Institute for Chemical Ecology in Jena, Germany. The results of this project are presented in this thesis.

In January 2008 she started working as a Public Relations official at the faculty of Biomedical engineering at the Technical University Eindhoven.

List of publications

van Leur H, Raaijmakers CE & van Dam NM (2006). A heritable glucosinolate polymorphism within natural populations of *Barbarea vulgaris*. *Phytochemistry* **67:** 1214-1223.

van Leur H, Vet LEM, van der Putten WH & van Dam NM (In Press). *Barbarea vulgaris* glucosinolate phenotypes differentially affect performance and preference of two different species of lepidopteran herbivores. *Journal of Chemical Ecology* (online DOI: 10.1007/s10886-007-9424-9)

van Leur H, Raaijmakers CE & van Dam NM (under review). Reciprocal interactions between the Cabbage root fly (*Delia radicum*) and two glucosinolate phenotypes of *Barbarea vulgaris*.

van Leur H, Kardol P, Raaijmakers CE, van der Putten WH, Vet LEM, ten Hooven FC, van Dam NM (manuscript). Interactions of *Barbarea vulgaris* glucosinolate phenotypes with above- and belowground invertebrate communities and their consequences for plant fitness.

van Leur H, Jansen JJ, Vet LEM, van der Putten WH, de Vos RCH, van Dam NM (manuscript). Metabolic and developmental costs associated with a *Barbarea vulgaris* glucosinolate polymorphism.

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: Hanneke van Leur
Date: 17 March 2008
Group: NIOO-KNAW, Department Multitrophic Interactions
Entomology & Nematology, Wageningen University and Research Centre

1) Start-up phase	date
► **First presentation of your project**	19 Nov 2003
Title: Glucosinolate polymorphism in B. vulgaris	
► **Writing or rewriting a project proposal**	30 Nov 2003
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD Student Day, Vrije Universiteit Amsterdam	03 Jun 2004
EPS PhD Student Day, Radboud University Nijmegen	02 Jun 2005
EPS PhD Student Day, Wageningen University	19 Sep 2006
► **EPS theme symposia**	
Theme 2 symposium 'Interactions between Plants and Biotic Agents', Utrecht University	17 Sep 2004
Theme 2 symposium 'Interactions between Plants and Biotic Agents', Leiden University	23 Jun 2005
Theme 2 symposium 'Interactions between Plants and Biotic Agents', University of Amsterdam	02 Feb 2007
► **NWO Lunteren days and other National Platforms**	
NWO-ALW 'Experimental Plant Sciences', Lunteren	04-05 Apr 2005
NWO-ALW 'Experimental Plant Sciences', Lunteren	03-04 Apr 2006
► **Seminars (series), workshops and symposia**	
MPI-ICE institute symposium 2004	31 Jan 2004
MPI-ICE seminar series on chemical ecology (16 seminars)	2003-2007
NIOO-KNAW institute seminar series (19 seminars)	2003-2007
KNAW symposium:"van bouwsteen tot biosfeer".	30 Sep 2004
BIORHIZ workshop1: "Direct interactions in the rhizosphere".	18 Jam 2005
Crucifer club wageningen	2005-2006
NIOO days Lunteren 2005	03-04 Mar 2005
NIOO days Lunteren 2006	01-02 Feb 2006
Workshop plant insect interactions	Apr 2006
NIOO days Lunteren 2007	15-16 Feb 2007
EPS symp. Ecology and Experimental Plant Sciences	03 Mar 2007
NWO Talentday	03 Apr 2007
► **Seminar plus**	
► **International symposia and congresses**	
IBC Vienna 2005 (Austria)	17-23 Jul 2005
ISCE Barcelona 2006 (Spain)	15-19 Jul 2006
International glucosinolate conference Jena 2006 (Germany)	11-14 Sep 2006
Symposium of Insect-plant relationships Uppsala 2007 (Sweden)	29 Jul-02 Aug 2007
► **Presentations**	
oral presentation MPI-CE, Jena, introducing project	20 Jan 2004
oral presentation MPI-CE, Jena, progression project	15 Jun 2004
2 oral presentations Entomology, Wageningen Crucifer club	19 Apr 2005
poster presentation IBC Vienna 2005	20 Jul 2005
oral presentation ISCE Barcelona 2006	19 Jul 2006
poster presentation 1st Int. Glucosinolate Conference Jena	10 Sep 2006
oral presentation ALW discussion group plant metabolites	06 Oct 2006
oral presentation EPS theme symposium 2006	02 Feb 2007
oral presentation SIP meeting Uppsala 2007	31 Jul 2007
► **IAB interview**	19 Sep 2006
► **Excursions**	
Subtotal Scientific Exposure	*23.4 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
Basic statistics (PE&RC)	Dec 2004
Survival analysis (PE&RC)	Feb 2005
Spring school:"Chemical communication: from gene to ecosystem".	Mar 2005
Workshop Metabolomics	May 2005
► **Journal club**	
MPI-ICE Darwin club meetings	2004
MPI-CE, Jena: Journal club	2004
Multitrophic Interactions Journal club	2003-2007
Population Biology of Plants Journal club	2005
PhD journal club NIOO	2005-2007
► **Individual research training**	
Training at the Max Planck Intstitute for Chemical Ecology 6 months	2004
Subtotal In-Depth Studies	*9.9 credits**

4) Personal development	date
► **Skill training courses**	
Training doelgericht werken en plannen (Boertien-trainingen)	Sep 2004
Presentation Skills (Centa)	Nov 2004
Scientific Writing for PhD (Centa)	Oct 2005
► **Organisation of PhD students day, course or conference**	
► **Memberschip of Board, Committee or PhD council**	
Member of the PhD council EPS	2004-2006
Member educational committee EPS	2006-2007
Subtotal Personal Development	*6.5 credits**

TOTAL NUMBER OF CREDIT POINTS*	**47.3**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*

The research presented in this thesis was conducted at the Department of Multitrophic Interactions at the Centre for Terrestrial Ecology of the Netherlands Institute of Ecology (NIOO-KNAW) in Heteren. This project was supported by the research council for Earth and Life Sciences (ALW) with financial aid from the Netherlands Organisation for Scientific Research (NWO), grant no. 813.04.005, and a VIDI grant, no. 864.02.001.

This is NIOO thesis 65.

Lay-out: Ron Galiart
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