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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, eight 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

1. 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 (Brown et al., 2003; Hartmann, 1996; Sang et al., 1984; Zhang et al., 1991). All this variability is restricted by the plant's genome (Cipollini et al., 2003; Kliebenstein et al., 2001a) 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, 2001). 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, because they even affect specialist herbivores (Agrawal and Kurashige, 2003). Nitriles and thiocyanates have a lesser toxicity to insects (Lambrix et al.,

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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.

2. 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*.

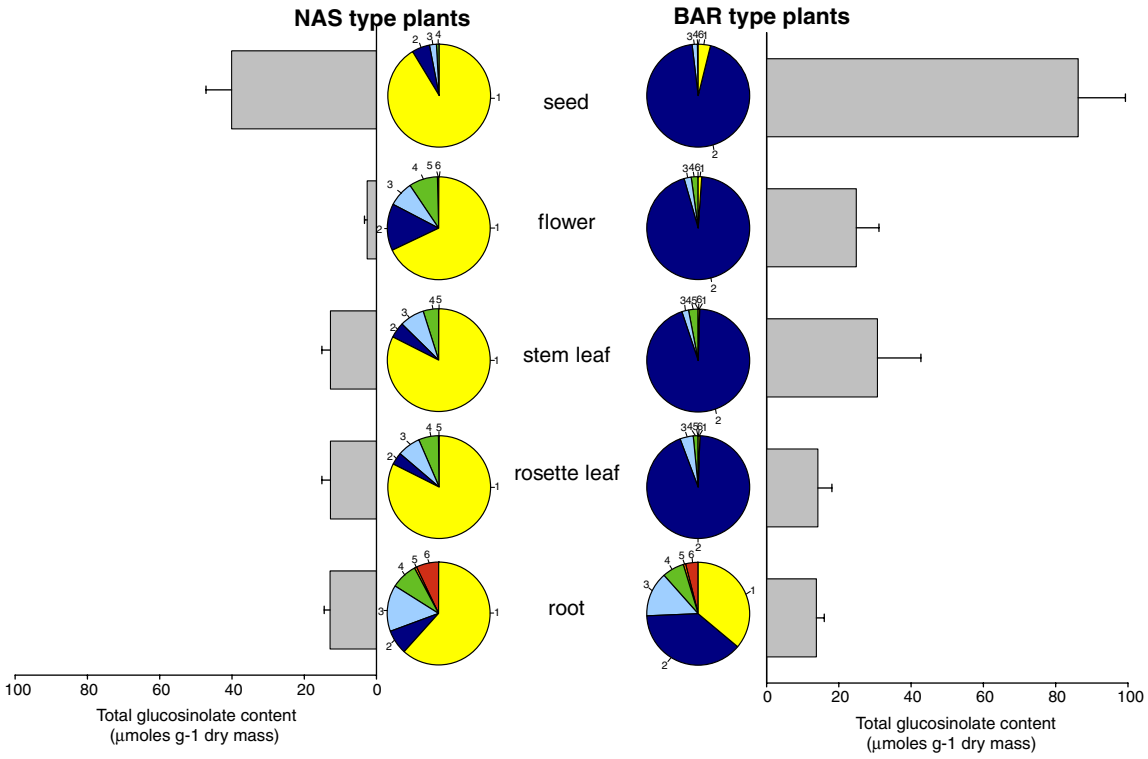

Fig. 1. The total glucosinolate levels (graphs, μmol dry mass + SE) and relative abundance of glucosinolates (pie charts, percentage of total glucosinolates) in seeds ($n = 18$ per type), roots, rosette leaves, stem leaves and flowers of NAS-type plants (left, $n = 3$) and BAR-type (right, $n = 3$) of *B. 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).

2.1. Glucosinolate profiles

Although the chemotypes contained the same set of glucosinolates they were clearly distinguished by the quantities of their main glucosinolate (Fig. 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 (Fig. 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 Fig. 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.

2.2. 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 (Fig. 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 (Fig. 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 (Fig. 2, letters over bars), which is consistent with the lower total glucosinolate levels in reproductive parts of NAS-plants (Fig. 1).

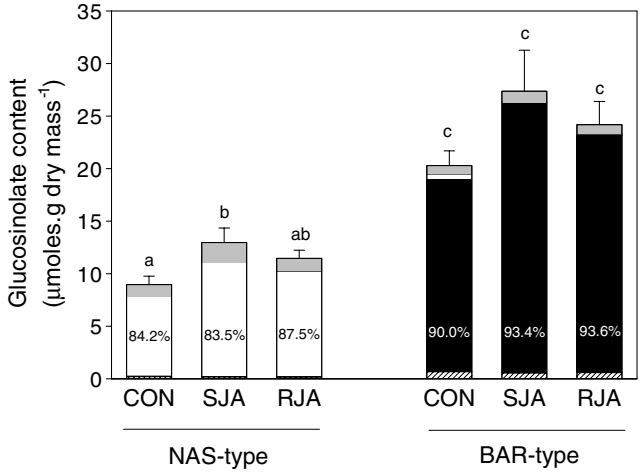

Fig. 2. Glucosinolate concentration in rosette leaves of *B. vulgaris* NAS or BAR plants treated with 500 μg jasmonic acid (JA) to their shoots (SJA) or to their roots (RJA). Controls (CON) were treated with acid water to control for effects of acid application. Error bars indicate standard error of 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.

2.3. 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 (Fig. 3 and Table 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.

2.4. 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

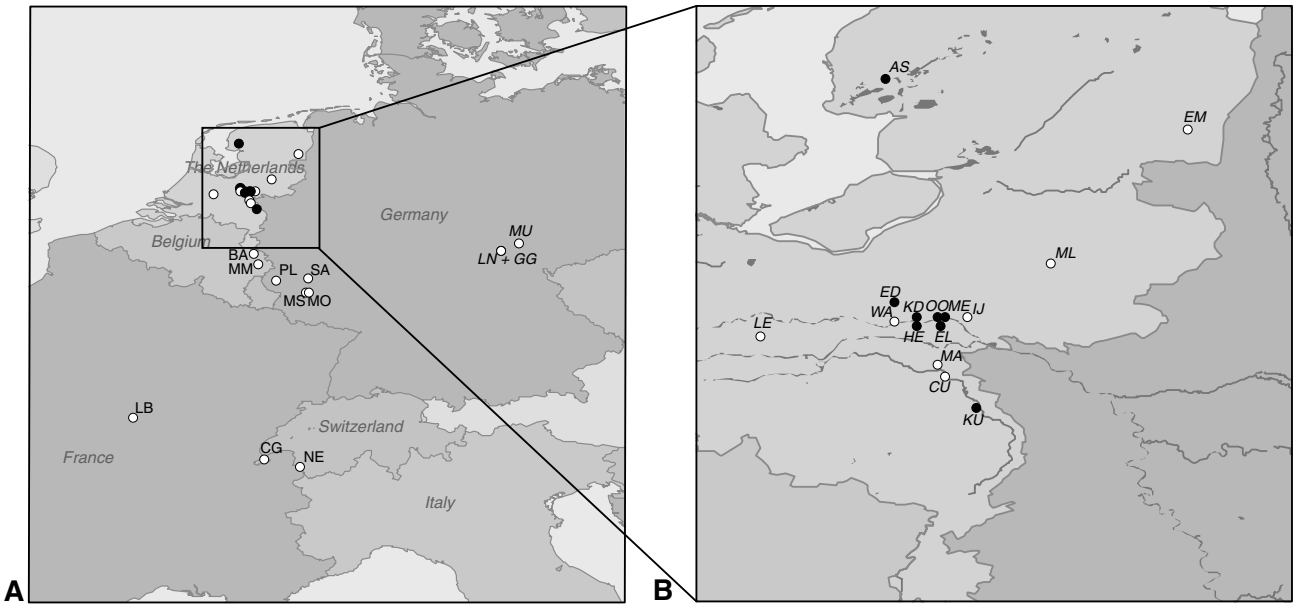

Fig. 3. Geographical distribution of sampled populations. Black spots are populations in which the chemotypes co-occur. White spots are the populations which contain solely BAR-type plants. Additional information about the populations is given in Table 1. (A) Overview of all sampled populations. (B) Dutch populations only.

Table 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.

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, 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 ($G_{\text{heterogeneity}} = 2.100$, $P = 0.717$; $G_{\text{pooled}} = 2.20$, $P = 0.698$; $G_{\text{total}} = 4.3$, $P = 0.366$; Table 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 ($G_{\text{pooled}} = 0$, $P = 1$ $G_{\text{total}} = 2.160$, $P = 0.706$; Table 2, cross 19–26). Additionally, we selected BAR-type plants of which selfings produced a uniform BAR-type offspring (Table 2, cross 27–28) suggesting that these plants were homozygous. When placed in our

Table 2
Expected and observed frequencies of both chemotypes in the offspring of controlled crosses

Cross	Origin	P	F1♀		F1♂	$F2_{\text{expected}}$		$F2_{\text{observed}}$		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

The origin refers to the natural population of the parent as described in Table 1 and depicted in and Fig. 2. In the columns P and F1 the numbers refer to the parent plant and the letters to the half-sib individuals.

experimental garden, which contained both BAR- and NAS-type plants, these plants indeed yielded only BAR offspring as well (Table 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.

3. Discussion

3.1. 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 defense 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 effects 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 (Agerbirk et al., 2003a; Kirkegaard and Sarwar, 1998; Sang et al., 1984; 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.

3.2. 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 (Agrawal and Kurashige, 2003; Li et al., 2000; Louda and Mole, 1991). 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.

3.3. 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). An 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). *A. 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.

3.4. 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. (2003a,b) 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 pubescent) 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).

3.5. 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).

4. Experimental

4.1. Plant rearing

For our experiments we used the crucifer *B. 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 $\mu mol\ m^{-2}\ s^{-1}$ 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.

4.2. 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 two *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 three 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.

4.3. 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 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 m 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.

4.4. 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 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. 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.

4.5. Inheritance of the chemotype

To check if the chemotype was heritable we selected nine pairs of plants (nine 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 ($n = 58 \pm 9.63$ 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 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 five NAS and six 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 two crosses succeeded. Both were selfings of BAR-type plants. (C) From the Oosterbeek population we screened and labelled 50 plants. Type determination revealed only one NAS plant. Seeds from this plant were collected, germinated and analysed. This yielded 80% BAR-type plants. Two BAR and two 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 h 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, cross 1–10). Subsequently, we crossed two heterozygous half-sibs (Table 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, cross 19–26). Finally we selfed BAR-type plants from the "Heteren" population (96% BAR) (Table 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, cross 29–30).

For statistical analyses of the crossing experiments we used the replicated goodness-of-fit test (*G*-statistic). $G_{\text{heterogeneity}}$ 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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