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The glucosinolate–myrosinase system in an ecological and evolutionary context

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Functional analysis of natural variation in the model species *Arabidopsis thaliana* has enabled the cloning of many glucosinolate biosynthesis and hydrolysis genes. Variation in these genes is central to understanding the ecological role of the glucosinolate–myrosinase defense system, and allows us to dissect the evolutionary and ecological forces that shape polymorphism at underlying loci. These same genes are also variable in other crucifer species, suggesting the presence of recurring selection, possibly mediated by insects. By utilizing the genomic tools available in *A. thaliana* to investigate these loci fully, it might be possible to generate detailed evolutionary or ecological models to apply to other species.

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Current Opinion in Plant Biology 2005, **8**:264–271

This review comes from a themed issue on
Physiology and metabolism
Edited by Toni Kutchan and Richard Dixon

Available online 25th March 2005

1369-5266/$ – see front matter
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DOI 10.1016/j.pbi.2005.03.002

Introduction

Crucifers and their major pre-formed chemical defenses, the glucosinolate-myrosinase system, have interested scientists and breeders for decades. The glucosinolate–myrosinase system's predominant role is in mediating the interaction of plants with their biotic environment. In addition to this ecological role, glucosinolates influence the flavor and/or health characteristics of agriculturally important Brassicaceous vegetable, oil and fodder crops. Consequently, there is considerable interest in manipulating glucosinolate composition in *Brassica* breeding programs. A general characteristic of the glucosinolate–myrosinase system is its variability at nearly every level, including biosynthesis, regulation, and breakdown, which is largely mediated by gene duplication and subsequent functional diversification. Analysis of glucosinolate natural variation in the model plant *Arabidopsis thaliana* and of the impact of this variation on resistance to herbivory has illustrated the complexity and ecological role of the glucosinolate–myrosinase system. This review focuses on recent progress in understanding functional, ecological and evolutionary aspects of cloned glucosinolate biosynthesis and hydrolysis genes.

Glucosinolate biosynthesis and breakdown

Glucosinolates are secondary metabolites in the Brassicaceae and related plant families. The glucosinolate core consists of a sulfonated oxime and a β-thio-glucose moiety. This core structure is linked to assorted side-chains that are derived from diverse amino acids, including alanine, leucine, isoleucine, tyrosine, tryptophan, valine, phenylalanine, methionine, and chain-extended homologs of methionine and phenylalanine. Methionine chain-extension occurs in a reaction cycle that follows an initial transamination of methionine to its corresponding 2-oxo acid. The reaction cycle consists of the condensation of the 2-oxo acid with acetylCoA, which is catalyzed by methylthioalkylmalate synthases [1,2], isomerization and oxidative decarboxylation. The resulting chain-extended 2-oxo acid can undergo additional chain-elongation cycles, each adding one further methylene group, or, following transamination, can enter the glucosinolate core biosynthetic pathway. The core pathway converts the amino acid to a *S*-alkylthiohydroximate via two consecutive reactions that are catalyzed by structurally specific cytochrome P450s, encoded by the *CYP79* and *CYP83* gene families (Table 1; [3–11,12•,13,14,15•,16]). *C-S* lyase activity results in the formation of thiohydroximates [17] that are converted to desulfo-glucosinolates by a non-specific *S*-glucosyltransferase [18]. The final glucosinolate is produced by sulfation by one of three structurally specific sulfotransferases (Table 1; [19]). Subsequently, various secondary side-chain modifications can occur, including oxidation, hydroxylation, alkenylation, acylation or esterification [20].

In plant herbivore defense, glucosinolates are activated by myrosinases, a specific class of β-thioglucosidases. *A. thaliana* and other species contain several myrosinase isoforms that form large enzyme complexes with myrosinase-binding and -associated proteins [21–24] of mostly unknown function. As the glucosinolates and myrosinases are stored in different compartments [25,26], tissue rupture is necessary to bring them into contact. Glucosinolate hydrolysis then forms different breakdown products that have variable bioactivities.

Natural variation in glucosinolate biosynthesis

The species *A. thaliana* contains more than thirty different glucosinolate structures, with the major glucosinolates

Table 1

Names and functions of *Arabidopsis* glucosinolate biosynthesis and hydrolysis proteins.

Name	[a]TAIR identifier	Function and notes	Reference(s)
MAM1	At5 g23010	Methylthioalkylmalate synthase: methionine carbon-chain extension, responsible for dihomo-methionine formation.	[1,2,41•]
MAM2	–	Methylthioalkylmalate synthase: methionine carbon-chain extension, responsible for homo-methionine formation, not present in Col-0.	[1]
MAML	At5 g23020	Methylthioalkylmalate synthase: methionine carbon-chain extension, responsible for long-chain methionine homologs.	[1] (see also [43])
CYP79A2	At5 g05260	Cytochrome P450: conversion of amino acids to aldoximes, acts on phenylalanine.	[3]
CYP79B2	At4 g39950	Cytochrome P450: conversion of amino acids to aldoximes, acts on tryptophan.	[4,5,15•]
CYP79B3	At2 g22330	Cytochrome P450: conversion of amino acids to aldoximes, acts on tryptophan.	[4,15•]
CYP79F1	At1 g16410	Cytochrome P450: conversion of amino acids to aldoximes, acts on homo- to pentahomo-methionine.	[6,9,12•,16]
CYP79F2	At1 g16400	Cytochrome P450: conversion of amino acids to aldoximes, acts on pentahomo- and hexahomo-methionine.	[6,9,12•,16]
CYP83A1	At4 g13770	Cytochrome P450: conversion of aldoximes to *S*-alkylthiohydroximates, acts on aliphatic aldoximes.	[13,14]
CYP83B1	At4 g31500	Cytochrome P450: conversion of aldoximes to *S*-alkylthiohydroximates, acts on aromatic and indole aldoximes.	[13,14]
C-*S* lyase	At2 g20610	Formation of thiohydroximates.	[17]
UGT74B1	At1 g24100	UDP-glucose:thiohydroximate *S*-glucosyltransferase: generation of desulfo-glucosinolates from thiohydroximates.	[18]
AtST5a	At1 g74100	Sulfotransferase: sulfation of desulfo-glucosinolates, acts preferably on tryptophan- and phenylalanine-derivatives.	[19]
AtST5b	At1 g74090	Sulfotransferase: sulfation of desulfo-glucosinolates, acts preferably on methionine-derivatives.	[19]
AtST5c	At1 g18590	Sulfotransferase: sulfation of desulfo-glucosinolates, acts preferably on methionine-derivatives.	[19]
AOP1	At4 g03070	2-oxoglutarate-dependent dioxygenase: unknown function.	[50]
AOP2	At4 g03060	2-oxoglutarate-dependent dioxygenase: formation of an alkenyl from a precursor methylsulfinyl side-chain.	[50]
AOP3	At4 g03050	2-oxoglutarate-dependent dioxygenase: formation of a hydroxyalkyl from a precursor methylsulfinyl side-chain.	[50]
TGG1	At5 g26000	Myrosinase: glucosinolate breakdown.	[21]
TGG2	At5 g25980	Myrosinase: glucosinolate breakdown.	[21]
TGG3	At5 g48375	Myrosinase: glucosinolate breakdown, pseudogene in Col-0.	[23]
ESP	At1 g54040	Epithiospecifier protein: determines glucosinolate breakdown product identity.	[52]

Abbreviation: TAIR, The *Arabidopsis* Information Resource.
[a] www.arabidopsis.org.

being derived from tryptophan and chain-extended methionine homologs. Within *A. thaliana*, however, ecotypes differ in both glucosinolate type and quantity [27,28]. The structural variation between ecotypes is due to genetic differences in elongation and in modification of methionine side chains. Genetic variability in the biosynthesis of the glucosinolate core structure could produce differences in glucosinolate accumulation, but such variability has not been identified to date.

Glucosinolate profiles are not determined solely by genetic fluctuations and respond to plant development and abiotic factors such as nitrogen, sulfur or potassium supply [29–33]. Glucosinolate gene expression and profiles also change in response to pathogen attack or herbivory [34,35•,36•]. Jasmonates, which contribute to signal transduction during insect herbivory, increase total glucosinolate concentration, primarily because of changes in indole glucosinolate concentration [29,36•,37–39]. Jasmonates also induce several aliphatic glucosinolates [29,37]. Salicylate, a pathogen attack signal, stimulates glucosinolate accumulation, either alone or in combination with other phytohormones [29,37], and may counteract jasmonate-mediated glucosinolate induction [29,37–39]. Most remarkably, *A. thaliana* ecotypes show regulatory polymorphism for the ability to induce glucosinolates in response to phytohormones [29].

Like glucosinolate biosynthesis, the expression of myrosinase, myrosinase-binding, and myrosinase-associated proteins varies among plant developmental stages and organs, and is differentially regulated upon phytohormone treatment or insect herbivory [21,22,30,40].

Variation in glucosinolate side-chain elongation

The *GS-Elong* locus in *Arabidopsis* [1,41•] and *Brassica* [42] encodes several methylthioalkylmalate synthases (MAM) and hence controls the side-chain length of methionine-derived glucosinolates. Allelic variation at *GS-Elong* influences glucosinolate composition and accumulation, as well as resistance to the generalist insect herbivore *Spodoptera exigua*, but does not detectably affect herbivory by the specialist lepidopteran *Plutella*

xylostella [41•]. In *A. thaliana*, *GS-Elong* displays extensive genetic complexity: the ancestral state contains three methylthioalkylmalate synthase genes, *MAM2*, *MAM1*, and *MAML*. MAM1 catalyzes the condensation reaction in the first and second cycles of methionine side-chain elongation [1,2], resulting in the addition of two methylene groups to form dihomo-methionine. Fine-mapping suggests that MAM2 catalyzes only the first condensation reaction, generating homo-methionine [1,41•], whereas MAML creates smaller quantities of longer side-chain methionines [43]. A sequence survey of *A. thaliana* ecotypes reveals that *MAM* gene composition at *GS-Elong* is shaped by gene duplication, deletion and conversion events [41•,44], consistent with a 'birth and death' model of *MAM* gene family evolution. In addition, balancing selection [45] was inferred in the evolution of the *MAM2* gene. Numerous other glucosinolate-related genes, including *CYP79F*s, *CYP79B*s, alkenyl/hydroxypropyl glucosinolate loci (*AOP*s), *A. thaliana SULFOTRANSFERASE5* (*AtST5*), and thioglucoside glucohydrolase (myrosinase) loci (*TGG*s), form tandemly repeated clusters, suggesting that similar evolutionary processes might have shaped these loci.

Variation in glucosinolate side-chain modification

Glucosinolates also have intra- and inter-specific variation in side-chain modification. In *Arabidopsis* and *Brassica*, this centers on whether the glucosinolate has a methylsulfinyl, alkenyl or hydroxyalkyl moiety [27,45–47]. The different compounds have diverse biological activities. For example, alkenyl glucosinolates are oviposition signals for cabbage webworm (*Hellula undalis*), whereas methylsulfinyl glucosinolates are anti-biotics [48,49]. These dissimilar biological effects might allow for variable or balancing selection, which could maintain the variation in side-chain structures.

In *A. thaliana*, the production of methylsulfinyl, alkenyl or hydroxyalkyl glucosinolates is controlled by a single genetic locus, *GS-AOP*. This locus has three alleles each of which controls the production of one side-chain type: *GS-ALK* produces alkenyl side-chains, *GS-OHP* produces hydroxyalkyl side-chains, whereas *GS*-null produces methylsulfinyl side-chains [46,50]. *GS-AOP* has been associated with generalist insect resistance, supporting the proposal that the different glucosinolate structures have dissimilar activities. In the *A. thaliana* ecotype Columbia, *GS-AOP* is comprised of two tandem genes, *AOP2* and *AOP3*. AOP2 produces alkenyl glucosinolates from a methylsulfinyl precursor, whereas AOP3 directs hydroxyalkyl production from the same methylsulfinyl moiety. *A. thaliana* ecotypes that contain foliar alkenyl glucosinolate express AOP2 but not AOP3 in their leaves, presumably because of a promoter mutation as AOP3 activity is detectable in the seeds [50]. By contrast, hydroxyalkyl-producing ecotypes express AOP3 in the seed and leaf but contain an unexpressed *AOP2* variant. Methylsulfinyl-accumulating ecotypes have polymorphisms that knock out AOP2 and block the expression of AOP3. F_1 plants from crosses between hydroxyalkyl and alkenyl accessions co-expressed AOP2 and AOP3, and accumulated hydroxyalkyl and alkenyl glucosinolates in the same leaf. However, none of 132 wild accessions accumulated both hydroxyalkyl and alkenyl glucosinolates. The absence of wild accessions that contain both glucosinolates could suggest that a mixture of these two glucosinolates is less beneficial than either one alone or could be the result of *A. thaliana*'s mainly self-fertilizing reproductive system.

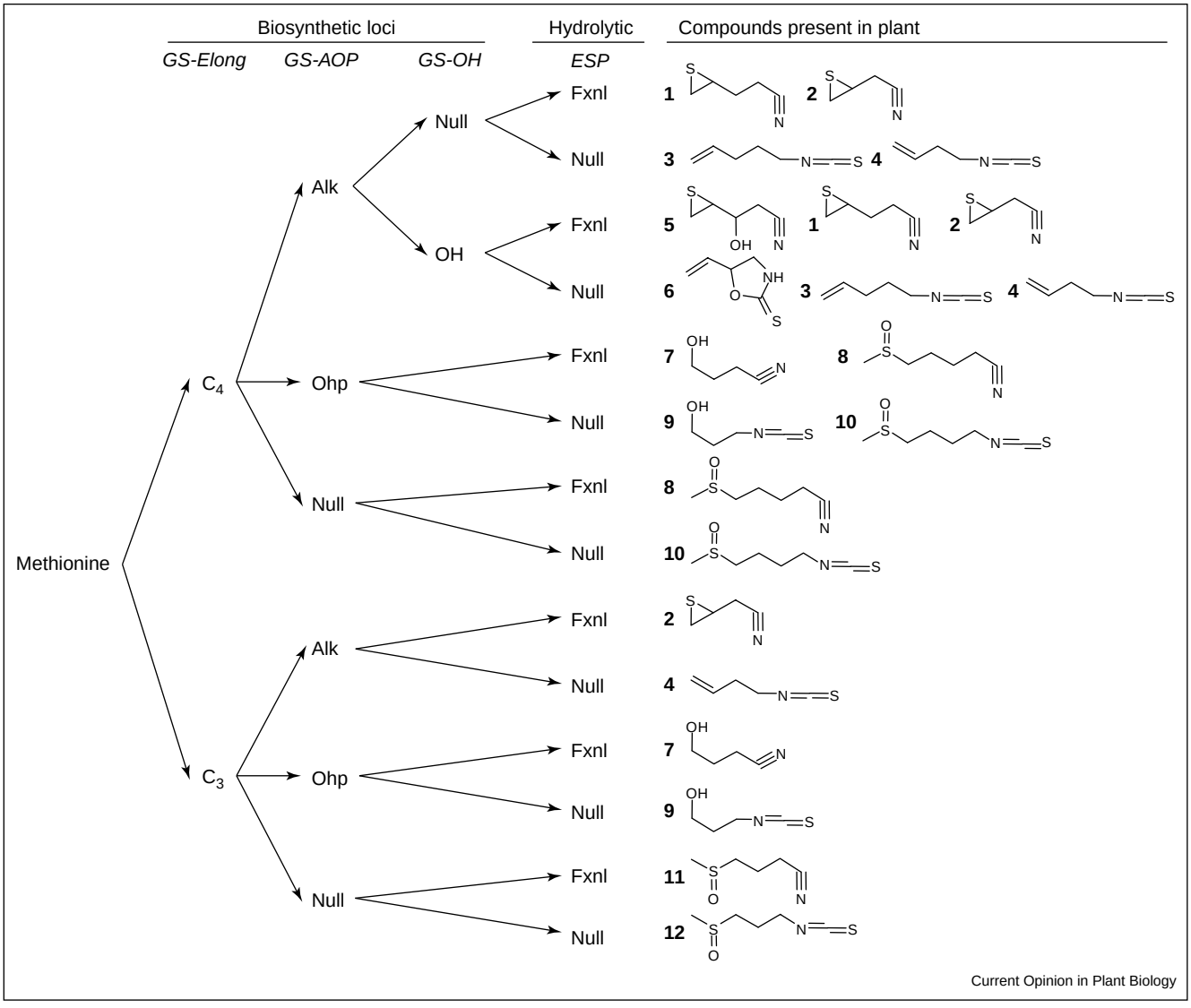
Numerous species, including *Brassica oleracea* and *Brassica napus*, show intra-specific variation in alkenyl and methylsulfinyl glucosinolate accumulation [47,51]. In *Brassica*, as in *Arabidopsis*, this variation is controlled by the *AOP2* homolog, with AOP2 directing alkenyl production and multiple independent loss-of-function variants causing methylsulfinyl accumulation. The fact that AOP2 directs the production of alkenyl glucosinolates in *Brassica* and *Arabidopsis* suggests that functional *AOP2* was maintained during speciation. Furthermore, it appears that there has been recurring selection for lines that accumulate methylsulfinylalkyl, as both genera have independent loss-of-function *AOP2* variants at moderate frequencies. The diverse biological activity of alkenyl and methylsulfinyl glucosinolates, combined with their parallel evolution in different species, suggests that fluctuating selection has influenced their evolution such that certain glucosinolates are favored in different environments. *GS-OH* is another naturally variable side-chain-modifying locus that is involved in hydroxylating alkenyl glucosinolates (Figure 1; [27,50]).

Variation in glucosinolate breakdown

Glucosinolates obtain their major biological activities following hydrolysis by myrosinase and associated proteins. Depending upon the composition and the activities of associated proteins, the myrosinase system can produce either isothiocyanate or nitrile/epithionitrile breakdown products [52]. Following myrosinase hydrolysis, nitriles and epithionitriles are generated by the epithiospecifier (ESP) protein from alkyl and alkenyl glucosinolates, respectively. Isothiocyanates form spontaneously when any glucosinolate is hydrolyzed in the absence of ESP. In *A. thaliana*, the production of nitriles as opposed to isothiocyanates is controlled by genetic variation at the *ESP* locus, with nitrile-producing ecotypes expressing functional ESP.

ESP interacts epistatically with *GS-Elong* and *GS-AOP* to influence defense against herbivore damage by the generalist insect herbivore *Trichoplusia ni* (Figure 2). The alkenyl isothiocyanates (GS-ALK/ESP–) and alkenyl epithionitriles (GS-ALK/ESP+) have equal defensive

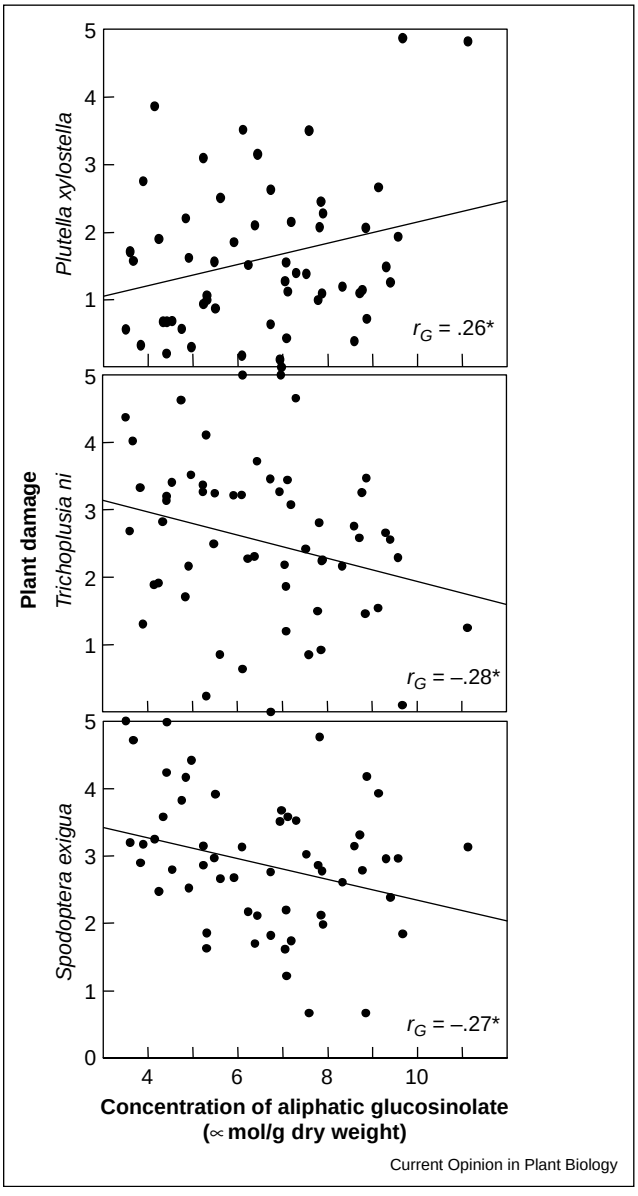
Figure 1



Epistatic control of glucosinolate structure. The interactions of known glucosinolate biosynthetic and hydrolytic loci are illustrated. The loci are listed at the top with their various genotypes below. The arrows represent a potential pathway through the different alleles at the various loci. The structures that would be synthesized by each path are shown to the right. The compounds are as follows: 1. epithiobutylnitrile, 2. epithiopropylnitrile, 3. but-3-enylisothiocyanate, 4. allylisothiocyanate, 5. epithio-3-hydroxy-butylnitrile, 6. goitrin, 7. 3-hydroxyproylpnitrile, 8. 4-methylsulfinylbutylnitrile, 9. 3-hydroxypropylisothiocyanate, 10. 4-methylsulfinylbutylisothiocyanate, 11. 3-methylsulfinylpropylnitrile, and 12. 3-methylsulfinylisothiocyanate. Alleles for the genetic loci are as follows: *GS-Elong* – C_3 = 3 carbon, C_4 = 4 carbon; *GS-AOP* – Alk = alkenyl glucosinolate, Ohp, hydroxyalkyl glucosinolate, and null = methylsulfinyl glucosinlate; *GS-OH* – OH = hydroxyalkenyl glucosinolate, null = no enzymatic activity; *ESP* – Fxnl = functional enzyme produces nitrile, null = no enzymatic activity.

capabilities that are greater than those of alkyl isothiocyanates (GS-OHP or null/ESP–) (Figure 1). For *T. ni*, the least repellent class of glucosinolate breakdown products are the alkyl nitriles (GS-OHP or null/ESP+) (Figure 1; [52]). These activity relationships are at the level of herbivory and do not take into account potential differences in oviposition or tritrophic activities that might help to limit damage to the plant. For example, nitriles are less effective oviposition signals than isothiocyanates and appear to play a role in attracting parasitic wasps [53]. Thus, selection within this system will depend upon whether an insect is best deterred at the level of oviposition, herbivory, or tritrophic interactions. If the biological effects of glucosinolates differ from one insect to another, this could result in a complex selection gradient that would change depending upon the local insect population. This level of complexity might be a widespread phenomenon in the Capparales, as there is extensive

Figure 2



Contrasting effects of glucosinolates on crucifer specialist and generalist insects. Plant damage and leaf aliphatic glucosinolate concentration are positively correlated for the crucifer specialist *Plutella xylostella*, and negatively correlated for the generalists *Trichoplusia ni* and *Spodoptera exigua*. This experiment was conducted with 58 *Arabidopsis* near-isogenic lines [41•] that display allelic variation at *GS–Elong* and *GS–AOP* and that harbor either the Colombia-0 (Col-0) or the Landsberg *erecta*-0 (*Ler*-0) alleles at these loci. Relative herbivory levels were quantified by estimating the percentage of leaf area consumed by the insects. RG, genetic regression of insect herbivory on line means for aliphatic glucosinolate concentration. *$p < 0.05$.

variation in glucosinolate hydrolysis throughout the clade [54].

Insects also impact plant–insect interactions by having the ability to alter the hydrolysis of ingested glucosinolates. Two crucifer specialist Lepidopterans have independently evolved mechanisms that deactivate the glucosinolate hydrolysis system. *Plutella xylostella* contains a sulfatase gut enzyme that removes the sulfate moiety, blocking myrosinase's ability to cleave the thioglucose [55]. The glucosinolates then pass through the insect's digestive tract as inactive desulfo-glucosinolates, allowing *Plutella* to feed on crucifers with relative impunity. By contrast, several generalist insects do not have glucosinolate sulfatase activities [55]. This agrees with the observation that no quantitative trait locus (QTL) for resistance to damage caused by *Plutella* herbivory was related to the glucosinolate-myrosinase system whereas seven of eight QTL for *Trichoplusia* resistance co-localize with glucosinolate QTL [56].

Another specialist lepidopteran, *Pieris rapae*, has also evolved a mechanism to reduce glucosinolate toxicity [57•]. *Pieris* utilizes an enzyme, nitrile-specifying protein (NSP), to direct the formation of nitriles instead of isothiocyanates during hydrolysis. NSP and ESP have similar activities but they have no sequence homology. In general, nitriles have lower toxicity than isothiocyanates, but nitriles have been implicated as key compounds in allowing parasitic wasps to identify *Arabidopsis* plants that are being attacked by Pierids [53]. Thus, it is possible that some tritrophic defenses might actually be controlled by NSP-mediated nitrile production.

In addition to insects that deflect glucosinolate hydrolysis, some species completely bypass myrosinase hydrolysis and accumulate intact glucosinolates [58]. Several of these insects have evolved their own myrosinase and use a bipartite glucosinolate-myrosinase system for their own defense [59].

Conclusions

Glucosinolate variation can dramatically impact a plant's fitness in response to attack by various pests. The primary genetic determinants of glucosinolate variation center on three biosynthetic loci and one hydrolytic locus that epistatically control the glucosinolate hydrolysis profile (namely *GS-Elong*, *GS-AOP*, *GS-OH* and *ESP*) (Figure 1). These loci are conserved across most Brassicaceae, but also exhibit parallel loss-of-function polymorphism. This pattern has been observed in *Arabis alpina*, *Arabidopsis petraea*, *A. thaliana* and *B. oleracea*. In *A. thaliana*, natural variation at all of these loci has been related to the differential control of lepidopteran herbivory, depending upon whether the insect is a crucifer generalist or specialist. Thus, it is possible that these evolutionary changes are due to the interactions of fluctuating insect populations with diverse glucosinolate profiles that have contrasting biological activities. The epistatic interaction between these loci means that the effects of a newly arising polymorphism in any of these genes could ripple

through the entire pathway, altering the potential fitness effects of enzymes encoded by the other genes.

The detection of epistatic interactions amongst these loci, as well as their complex genetic structure, will make it interesting to compare single and multi-locus, as well as genetic and phenotypic, models of evolution and ecology. Studies are currently underway to generate transgenic *A. thaliana* lines that specifically vary in each of these steps to verify their epistatic and phenotypic effects on both glucosinolate structure and herbivory. The genomic, functional and phenotypic information obtained on this pathway from growth-chamber experiments with *A. thaliana* should greatly enhance future efforts to understand how glucosinolate variation impacts plant–insect interactions and plant fitness in their natural context.

Acknowledgements

This work was supported by the Max Planck Society, and by grants to T Mitchell-Olds from the European Union (Contract QLRT-2000-01097), to T Mitchell-Olds and DJ Kliebenstein from the US National Science Foundation (Grants DEB-9527725 and MCB-0323759), and to J Kroymann from the Deutsche Forschungsgesellschaft (KR 2237/2 1). DJ Kliebenstein and J Kroymann contributed equally to this work.

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