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Gene expression and glucosinolate accumulation in *Arabidopsis thaliana* in response to generalist and specialist herbivores of different feeding guilds and the role of defense signaling pathways

Inga Mewis [a,b,c,*], James G. Tokuhisa [b,1], Jack C. Schultz [a], Heidi M. Appel [a], Christian Ulrichs [c], Jonathan Gershenzon [b]

[a] *Pennsylvania State University, Department of Entomology, Chemical Ecology Laboratory, State College, PA 16802, USA*
[b] *Max Planck Institute for Chemical Ecology, Department of Biochemistry, Hans-Knöll-Strasse, 8, 07745 Jena, Germany*
[c] *Humboldt University Berlin, Institute for Horticultural Science, Section Urban Horticulture, Lentzeallee 55-57, 14195 Berlin, Germany*

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Abstract

Glucosinolate accumulation and expression of glucosinolate biosynthetic genes were studied in response to four herbivores in *Arabidopsis thaliana* (L.) wild-type (Columbia) and mutant lines affected in defense signaling. Herbivory on wild-type plants led to increased aliphatic glucosinolate content for three of four herbivores tested, the aphid generalist *Myzus persicae* (Sulzer), the aphid specialist *Brevicoryne brassicae* (L.), and the lepidopteran generalist *Spodoptera exigua* Hübner. The lepidopteran specialist *Pieris rapae* L. did not alter aliphatic glucosinolate content in the wild-type, but indole glucosinolates increased slightly. Gene expression associated with aliphatic glucosinolate biosynthesis increased after feeding by all species, indicating that glucosinolate accumulation is not always regulated at the level of these gene transcripts. *A. thaliana* lines with mutations in jasmonate (*coi1*), salicylate (*npr1*), and ethylene signaling (*etr1*) diverged in gene expression, glucosinolate content, and insect performance compared to wild-type suggesting the involvement of all three modes of signaling in responses to herbivores. The *coi1* mutant had much lower constitutive levels of aliphatic glucosinolates than wild-type but content increased in response to herbivory. In contrast, *npr1* had higher constitutive levels of aliphatic glucosinolates and levels did not increase after feeding. Glucosinolate content of the *etr1* mutant was comparable to wild-type and did not change with herbivory, except for *P. rapae* feeding which elicited elevated indolyl glucosinolate levels. Unlike the wild-type response, gene transcripts of aliphatic glucosinolate biosynthesis did not generally increase in the mutants. Both glucosinolate content and gene expression data indicate that salicylate and ethylene signaling repress some jasmonate-mediated responses to herbivory.

Keywords: *Arabidopsis*; Glucosinolate biosynthesis; Insect herbivory; Plant response; Transcript levels

1. Introduction

Many plants respond dynamically to herbivore attack by increasing levels of anti-herbivore defenses. *Arabidopsis thaliana* (L.) alters its production of secondary metabolites, including glucosinolates (GS), phenolics, and terpenoids, in response to attack by insects and microbes (Halkier, 1999; Madhuri and Reddy, 1999; Harrewijn et al., 2001; Mikkelsen et al., 2003; D'Auria and Gershenzon, 2005). GS are defense compounds found exclusively in plants of the order Brassicales (formerly Capparales), including agriculturally important crop plants of the Brassicaceae family (Halkier and Gershenzon, 2006). These compounds are stored separately from specific hydrolytic enzymes known as myrosinases that catalyze the formation of biologically active

* Corresponding author. Address: Humboldt University Berlin, Institute for Horticultural Science, Section Urban Horticulture, Lentzeallee 55-57, 14195 Berlin, Germany. Tel.: +49 30 31471351; fax: +49 30 31471178.
E-mail address: inga@entomology.de (I. Mewis).
[1] Virginia Tech University, Horticulture Department, Blacksburg, VA 24061, USA.

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compounds such as isothiocyanates and nitriles (Bones and Rossiter, 1996; Rask et al., 2000). While the GS/myrosinase system comprises a defense against generalist insects, pathogens, bacteria, and other herbivores, many specialist insects have adapted to glucosinolates and corresponding hydrolysis products and use them in finding and accepting their host plants (Giamoustaris and Mithen, 1995; Tierens et al., 2001; Renwick, 2002).

The 120 different GS identified to date share a common core structure of a β-D-thioglucose group linked to a sulfonated aldoxime moiety. They differ in their aglycone side chain (Fahey et al., 2001). The source of the side chain defines three major classes of GS: aliphatic GS are derived in *A. thaliana* principally from Met, indolyl GS from Trp, and aromatic GS from Phe.

Because we were interested in the molecular bases for the specific changes in GS profiles of the ecotype Columbia caused by specific insect feeding, the present study focused on the expression of genes related to pathways for indolyl and aliphatic GS biosynthesis. The biosynthesis of GS can be divided into three separate phases: (1) insertion of methylene groups (chain elongation) into the side chains of certain aliphatic and aromatic amino acid precursors, (2) reconfiguration of the amino acid moiety in core GS biosynthesis, and (3) further modification of side chains. Phases one and two of GS biosynthesis in *A. thaliana* are depicted in Fig. 1. Highlighted are one step in the chain elongation pathway of methionine catalyzed by methylthioalkylmalate synthases (*MAM1* and *MAM3*/*MAM-L*, *GS-ELONG*), aldoxime formation from Met, Phe, and Trp precursors catalyzed by the CYP79 cytochrome P450 monoxygenase family, aldoxime oxidation by members of the CYP83 family of cytochrome P450 monoxygenases, thiohydroximic acid formation by C-S-lyase, desulfo-GS formation by an *S*-glycosyltransferase (UGT), and finally GS formation by sulfotransferases (Field et al., 2004; Piotrowski et al., 2004; Kliebenstein et al., 2005; Grubb and Abel, 2006; Halkier and Gershenzon, 2006).

Five *CYP79* cytochrome-P450s use various amino acid substrates to catalyze aldoxime formation for GS biosynthesis in *A. thaliana*. CYP79A2 uses phenylalanine as a substrate. The CYP79F subfamily metabolizes carbon chain-elongated derivatives of methionine with one to six (CYP79F1) or five to six (CYP79F2) additional methylene groups in their side chain (Chen et al., 2003). Tryptophan is the substrate for CYP79B2 and CYP79B3 that contribute to indolyl GS biosynthesis. Mutants with lesion in both of these genes lack indolyl GS (Zhao et al., 2002). *CYP79B2* and *CYP79B3* have been shown to be induced by the pathogen *Erwina carotovora* (Jones), but treatment with jasmonic acid (JA) changed only expression levels of *CYP79B3* (Brader et al., 2001). The primary function of CYP83A1 is the oxidation of nitrogen atoms of aliphatic aldoximes (Hemm et al., 2003). The homologous reaction for indole and aromatic aldoximes is catalyzed by CYP83B1. However, defects in CYP83B1 activity, *sur2* mutant, as well as loss of functional CYP83A1, *ref2* mutant, did not lead to complete loss of one or the other GS class, indicating that both enzymes can metabolize all aldoximes (Bak and Feyereisen, 2001; Hemm et al., 2003).

Indolyl GS are widely distributed in the Brassicaceae family including all *A. thaliana* ecotypes, where their abundance is influenced strongly by environmental factors (Kliebenstein et al., 2001a; Review in: Raybould and Moyes, 2001). On the other hand, aliphatic GS composition in *A. thaliana* ecotypes and *Brassica* species is highly variable and considered to be under strong genetic control (Kliebenstein et al., 2001a,b; Li and Quiros, 2002). So far little attention was paid to the influence of different environmental factors on aliphatic GS production and the eco-

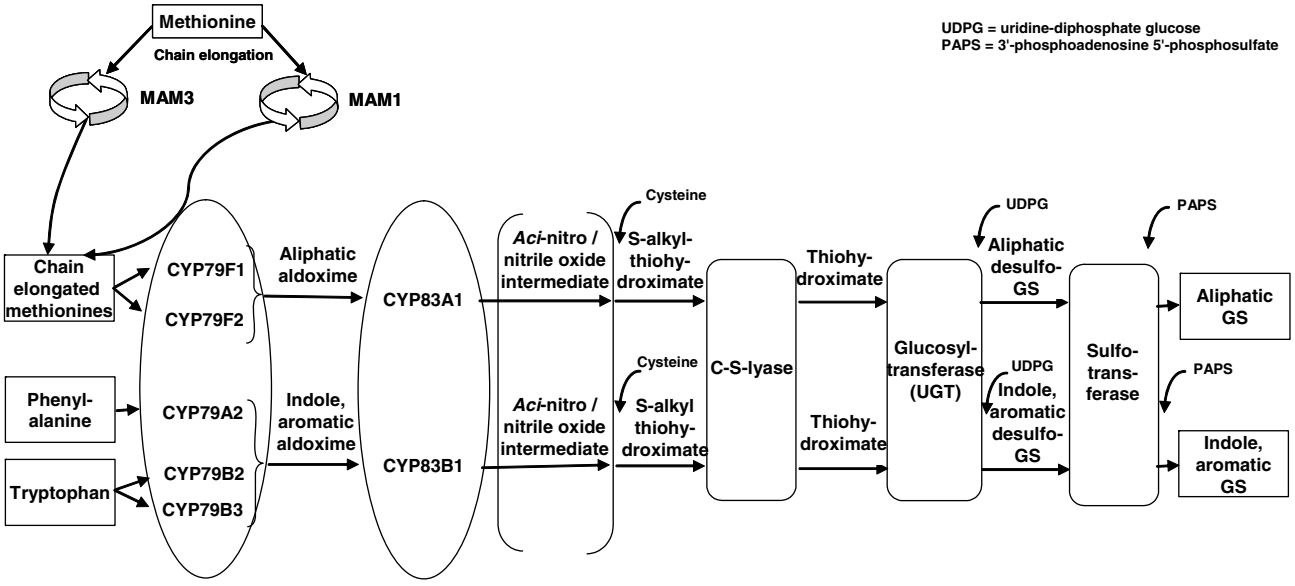

Fig. 1. Outline of glucosinolate biosynthetic pathways in *A. thaliana*. Expression studies were performed on genes encoding enzymes for each step, except *CYP79F2*, *CYP79A2*, C-S-lyase, and sulfotransferase (broad arrows represent multiple enzymes).

logical significance of the aliphatic GS diversity for insect resistance. The aromatic GS of *A. thaliana* are of low abundance and found predominantly in the seeds (Kliebenstein et al., 2001b) and they are therefore, not relevant to the present study which focuses on leaf chewing and phloem feeding insects.

Different signal transduction cascades mediate plant responses to various stresses. Responses to mechanical wounding or feeding by chewing insects involve the octadecanoid signaling pathway via JA and related compounds (Titarenko et al., 1997; Kessler and Baldwin, 2002). The JA pathway appears to involve and/or require ethylene (ET) for some responses to insects and pathogens (Penninckx et al., 1998). The benzenoid signaling molecule salicylic acid (SA) is crucial for local hypersensitive response and systemic acquired resistance against many pathogens (Glazebrook et al., 2003). All three signaling molecules, JA, SA, and ET, are believed to fine tune the plant defense response to different stressors, whereby the pathways can function synergistically as well as antagonistically (Stotz et al., 2000; Kunkel and Brooks, 2002; Cui et al., 2002; Glazebrook et al., 2003).

Indolyl GS accumulation in *A. thaliana* and *Brassica napus* L. is documented to be induced by mechanical wounding, insect feeding, and treatment with JA, or SA (Bodnaryk, 1992; Kiddle et al., 1994; Hopkins et al., 1998; Bartlet et al., 1999; Kliebenstein et al., 2002; Mikkelsen et al., 2003). Fewer studies have reported the induction of aliphatic or aromatic GS (Kiddle et al., 1994; Kliebenstein et al., 2002; Mikkelsen et al., 2003). The transcript levels for some genes related to indolyl and aliphatic GS biosynthesis in *A. thaliana* have been shown to increase following mechanical wounding and treatment with JA and this corresponds with increased levels of indolyl GS (Mikkelsen et al., 2003). Reymond et al. (2000) revealed in a microarray study differences between *Pieris rapae* L. – attacked and mechanically-wounded *A. thaliana* plants, particularly in the expression of dehydration-inducible genes. Recently, Reymond et al. (2004) identified more than 100 *P. rapae*-responsive genes potentially involved in defense, including genes involved in pathogenesis, indolyl GS biosynthesis (*CYP79B2* and *CYP83B1*), and signal transduction, most of which were induced rather than repressed. Furthermore, the authors compared expression levels following feeding by the crucifer specialist *P. rapae* with those by the generalist *Spodoptera littoralis* Boisduval. The expression patterns were surprisingly similar given the distinct salivary components of the two herbivores (Alborn et al., 2003). A transcript profiling of *A. thaliana* leaves following phloem feeding by aphids indicated the activation of the JA signaling pathway along with the SA pathway induced as strongly as that associated with pathogen infection (Moran and Thompson, 2001). Given the vast differences between phloem-feeding insects and chewing/cell damaging feeders such difference in plant response is not surprising.

The distinct defense responses of *A. thaliana* to different feeding specialist herbivores has not been compared explicitly in a single study, despite evidence that these insects may interact with signaling pathways differently, which should result in different gene expression profiles and patterns of GS production. Our previous study, Mewis et al. (2005), focused on the host plant resistance of *A. thaliana* to three insects and GS accumulation in signaling mutants. In the current study, we investigate the linkage between GS accumulation and the expression of GS biosynthesis and signaling pathway genes in response to herbivory. To compare the effects of specialist versus generalist herbivores, as well as different feeding guilds, we studied two species of chewing insects, the specialist caterpillar *P. rapae* and the generalist caterpillar *Spodoptera exigua* Hübner, and two phloem feeders, the specialist aphid *Brevicoryne brassicae* (L.) and the generalist aphid *Myzus persicae* (Sulzer). To our knowledge this is the first study presenting insect specific induced gene expression and changes in GS chemistry in *A. thaliana* linked to ecological outcomes.

2. Results

2.1. Feeding by aphids and lepidopterans had different effects on glucosinolate content in Columbia wild-type

Because very little is known about the specific biochemical response to different herbivorous insects in *A. thaliana*, we first compared the GS levels and specific gene expression in Columbia wild-type (Col WT) elicited by two aphid and lepidopteran species. Insect treatments were designed to result in equal amounts of plant damage within each insect guild by using the same number of aphids or the same developmental stage of lepidopteran. Aphids were maintained on the plants for one week and caterpillars for three days. Here, we observed different elicited GS response in *A. thaliana* Col WT depending on insect guild and species (Fig. 2a and b).

Feeding by both, the generalist aphid *M. persicae* and the specialist aphid *B. brassicae*, increased the aliphatic GS content of Col WT rosettes about 20% compared to the control (Fig. 2a). Only the short chain compounds, 3-methylsulfinylpropyl GS and 4-methylsulfinylbutyl GS, increased significantly (Tukey's HSD test $p \leqslant 0.05$). Under these conditions, the only change, an increase in indolyl GS content, was detected after feeding by *M. persicae* (Fig. 2a). Among the lepidopterans, much greater differences were found for specialist and generalist feeders (Fig. 2b). Three days of feeding by the generalist *S. exigua* led to the significant accumulation of both short and long (8-methylsulfinyloctyl GS, $p = 0.06$) chain aliphatic GS by more than 50% (Fig. 2b). In addition, indolyl GS increased twofold. In contrast, a similar amount of feeding by the specialist *P. rapae* did not cause any induction of aliphatic GS, while the indolyl GS content increased by about 20%, with the increase mainly due to additional 1-methoxyindol-3-ylmethyl GS (Fig. 2b).

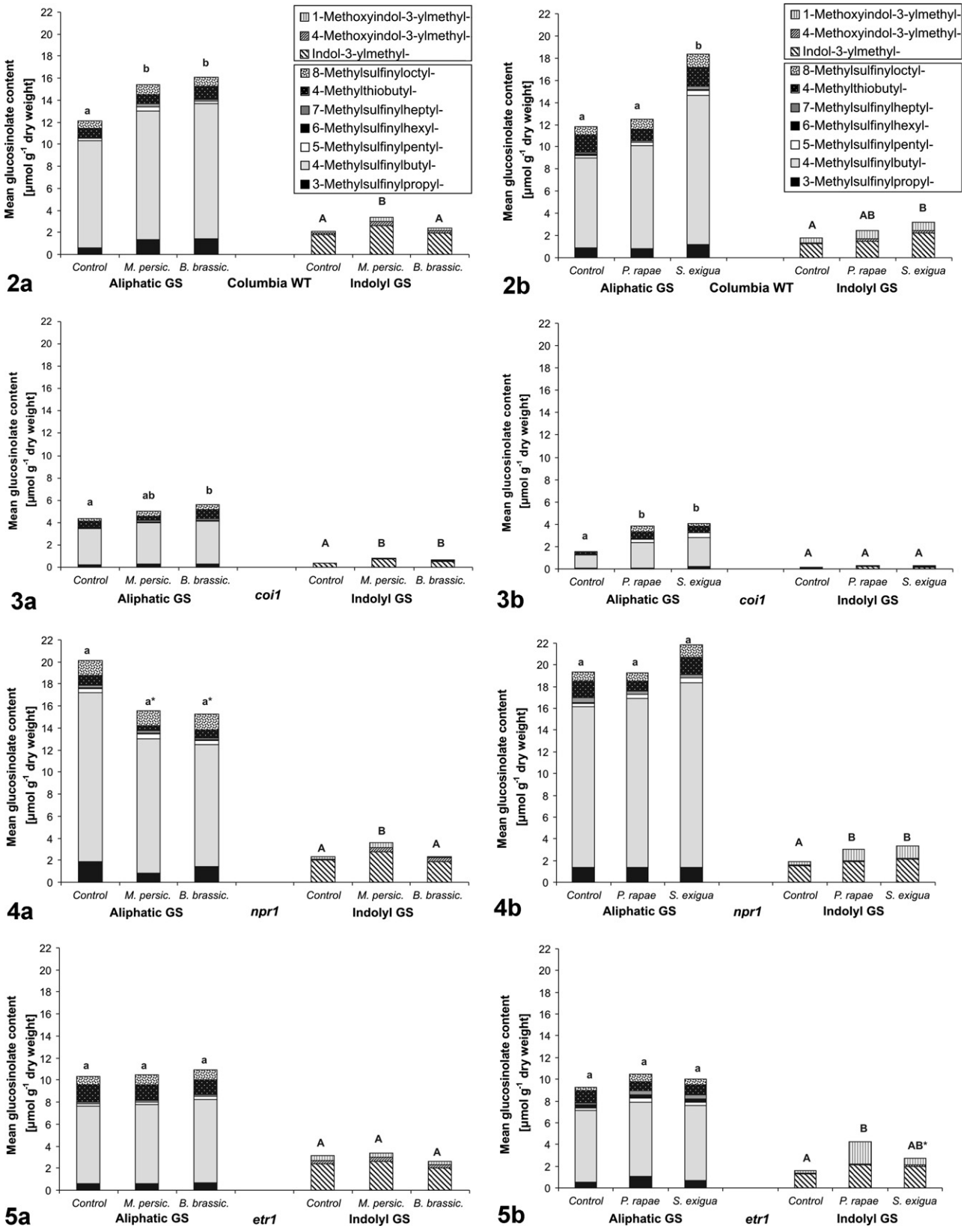

Fig. 2, 3, 4, and 5a, b. Glucosinolate content of *A. thaliana* Columbia wild-type (2) and signaling mutants *coi1* (3), *npr1* (4), and *etr1* (5) after one week aphid feeding on 40-day-old plants (a), and after three days feeding of 2nd instar caterpillars on 42-days-old plants (b). Controls show glucosinolate content with no feeding. (Different letters indicate significant differences with Tukey's HSD test: $p \leqslant 0.05$, $^{*}\, p < 0.1$. *M. persic.*, *M. persicae*; *B. brassic.*, *B. brassicae*).

2.2. Modification of signaling pathways influenced both glucosinolate accumulation and insect performance

Various *A. thaliana* signaling pathway mutants were used to determine which pathways mediate changes in GS accumulation by herbivores. Experiments were carried out with *coronatine insensitive 1* (*coi1*), non-expressor of PR-1*npr1*, and *ethylene resistant 1* (*etr1*) mutant lines, which are blocked in jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) signaling, respectively. In the absence of insect feeding, *coi1* showed marked decreases in overall GS levels compared to Col WT (Figs. 2 and 3). In contrast, *npr1* plants exhibited higher amounts of total aliphatic GS compared to Col WT due especially to an increase of 4-methylsulfinylbutyl GS (Figs. 2 and 4). The *etr1* plants contained similar GS levels as the wild-type (Figs. 2 and 5).

The *coi1* mutation changed the plant response to the different insects. Total aliphatic and indolyl GS content was higher than in control plants after one week exposure to the aphids *M. persicae* and *B. brassicae* (Fig. 3a). In contrast to results with Col WT, aliphatic but not indolyl GS increased significantly after feeding by both lepidopterans, *P. rapae* and *S. exigua* (Fig. 3b). The methylsulfinyl- and 4-methylthiobutyl-GS content increased about fourfold after caterpillar feeding compared to the *coi1* control, while in Col WT (also *npr1* and *etr1*) this compound decreased after feeding of the specialist caterpillar. In *npr1* plants, aliphatic GS decreased after feeding by the aphids *M. persicae* and *B. brassicae* instead of increasing as in Col WT (Fig. 4a). Lepidopteran feeding resulted in no significant changes in aliphatic GS levels in *npr1*, though *S.exigua* feeding had resulted in significant change in Col WT (Fig. 4b). But increases in indolyl GS were significant after lepidopteran feeding in *npr1*. In contrast to Col WT and other mutants, aphid feeding on *etr1* plants produced no change in GS (Fig. 5a). Caterpillar feeding also had little effect on aliphatic GS content (Fig. 5b). However, indolyl GS contents did increase in *etr1*, particularly in response to *P. rapae* feeding. With *P. rapae*, 1-methoxyindol-3-ylmethyl GS increased about tenfold in *etr1* compared to twofold increases in Col WT.

We also assessed the insect performance on the various signaling pathway mutants. On Col WT, the population size of aphids increased about fourfold within one week for *M. persicae* and threefold for *B. brassicae* (Fig. 6a). However, aphid numbers of both, specialist and generalist, species on *coi1* were significantly higher than on Col WT, and those on *npr1* and *etr1* were significantly lower than on Col WT (Fig. 6a). Among the lepidopterans, *P. rapae* 2nd instar performance showed a similar pattern to that of the aphids with less weight gain per leaf area consumed on *npr1* and *etr1* than on Col WT (Fig. 6b). On the other hand, *S. exigua* performance on *npr1* and *etr1* was not different from Col WT, but there was significantly more weight gain per leaf area eaten on *coi1* (Fig. 6b), which has a very low GS level.

2.3. Insect feeding increased transcript levels of many glucosinolate biosynthetic genes in Columbia wild-type

Insect feeding on Col WT led to changes in transcript levels of some genes of GS biosynthesis (Tables 1 and 2). There were significant increases in expression levels of genes of aliphatic GS biosynthesis, such as *MAM1* and *CYP79F1* (Table 1) but genes of indolyl GS biosynthesis, such as *CYP79B2*, *CYP79B3*, and *CYP83B1*, were generally not affected. These results correspond to the increases in aliphatic GS contegnt observed after feeding by these three insect species (Fig. 2a and b). On the other hand, feeding by the specialist caterpillar *P. rapae* induced the aliphatic GS gene *MAM1* but not *CYP79F1*, while the transcript levels of other genes of indolyl GS biosynthesis (*CYP79B3* and *CYP83B1*) increased significantly compared to the control plants (Table 1). Yet, there was only a slight increase in indolyl GS content after feeding of *P. rapae* (Fig. 2b).

We also measured the transcript levels of other genes commonly induced by insect feeding and defense signaling. Expression levels of *HEL* (PR4, induced by insect

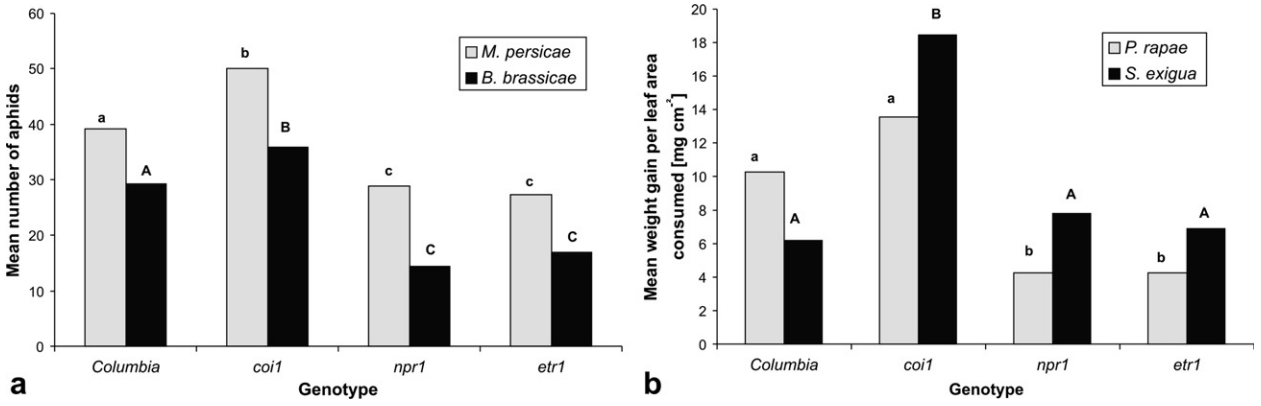

Fig. 6a, b. Insect performance on *A. thaliana* signaling mutants in comparison to Columbia wild-type: (a) Population size of aphids after one week (initially 10 per plant) and (b) weight of 2nd instar caterpillars related to leaf area eaten after three days. (Different letters indicate significant differences at $p \leqslant 0.05$ within one species, Fisher's LSD test.)

Table 1
Insect-induced changes in transcript levels of glucosinolate biosynthetic genes in *A. thaliana* Columbia wild-type after one week of aphid feeding (plants 40 days old) or three days of caterpillar feeding (plants 42 days old)

Gene	AGI Code No	Description	Mean change in transcript level (assigned ranks)			
			Aphid experiment		Caterpillar experiment	
			M. persicae	*B. brassicae*	*P. rapae*	*S. exigua*
Glucosinolate biosynthesis genes						
MAM3(MAM-L)	At5g23020	Methylthioalkylmalate synthase 3	++	$++^{**}$	++	++
MAM1	At5g23010	Methylthioalkylmalate synthase 1	$++^{**}$	$++^{**}$	$++^{**}$	$++^{**}$
CYP79F1	At1g16410	Cytochrome P450 79F1	$++^{*}$	$++^{**}$	+/−	$+^{*}$
CYP79B2	At4g39950	Cytochrome P450 79B2	+/−	$+^{**}$	$+^{**}$	+/−
CYP79B3	At2g22330	Cytochrome P450 79B3	+/−	+/−	$++^{**}$	+
CYP83A1	At4g13770	Cytochrome P450 83A1	+/−	+/−	$++^{**}$	+/−
CYP83B1	At4g31500	Cytochrome P450 83B1	+	+/−	$+^{**}$	+/−
UGT	At1g24100	*S*-glucosyltransferase	+	$+^{**}$	$+^{*}$	$+^{*}$
Selected genes commonly induced by defense signaling						
HEL/PR4	At3g04720	Hevein-like protein	+/−	+/−	$+^{*}$	+/−
BGL1	At1g52400	β-Glucosidase 1	+/−	+/−	$+^{*}$	+/−
CaEF	At2g33380	Calcium-binding RD20 protein	+/−	+/−	$++^{**}$	$++^{*}$
PR1	At2g14610	Pathogenesis related protein 1 precursor	$++^{*}$	$++^{**}$	$++^{**}$	$++^{**}$

A selection of genes commonly induced by defense signaling is also included.
++, ⩾ 2-fold increase; +, 1.5–2 fold increase; +/−, no change; −, 1.5–2 fold decrease; and − −, ⩾2-fold increase; Asterisks indicate significant differences in transcript levels between insect treatments and control plants by Kruskal–Wallis test: $^{**}p \leqslant 0.05$ and $^{*}p \leqslant 0.1$.

feeding) and *BGL1* (commonly induced by JA) increased significantly only after feeding by *P. rapae*, while transcript levels of *CaEF*, a gene which responds to ET were increased by caterpillar but not by aphid feeding (Table 1). *JMT*, a gene induced by mechanical wounding, did not show changes in transcript level after insect treatments (data not shown), while expression of *PR1* (associated with the SA pathway), was induced by both aphids and caterpillars (Table 1).

2.4. Insect feeding on signaling pathway mutants had divergent effects on gene expression

Changes in gene transcript levels in signaling pathway mutants were analyzed in a separate experiment with aphid and *S. exigua* feeding. Changes in GS content obtained in this experiment (data not shown) corresponded closely to that of the presented data set. For all signaling mutants, the transcript pattern differed from that of Col WT (Table 2).

The *coi1* mutation lead to decreases in the transcript levels of most analyzed genes after insect feeding (Table 2). This was especially true for genes of aliphatic GS biosynthesis, such as *MAM1/3* and *CYP79F1*, which mostly increased after insect treatments on Col WT. After insect feeding on *npr1* mutants, few changes in transcript pattern were observed compared to the untreated control. Transcript levels of genes of aliphatic GS biosynthesis, such as *MAM1*, *CYP79F1*, and *CYP83A1*, were equally abundant in all treatments, but *UGT* decreased after feeding by *M. persicae* and *B. brassicae*. The transcript pattern in *etr1* also differed from Col WT after insect feeding. There was much less increase in levels of genes of aliphatic GS. However, aphid feeding but not *S. exigua* feeding induced transcripts of *CYP79B2* and *UGT*. Interestingly, the transcript level of *HEL* decreased after feeding by *S. exigua* on *etr1*.

3. Discussion

3.1. Increased transcript levels of glucosinolate biosynthetic genes after herbivory do not always lead to increases in glucosinolate accumulation

Very little is known about specific molecular and biochemical responses of plants confronted by specialist vs. generalist herbivores of different feeding guilds. We measured GS accumulation and transcripts of GS biosynthetic genes in Col WT rosettes after feeding by two aphid and two lepidopteran species, a specialist and generalist of each type, and found some striking differences. Feeding by both aphids, the specialist *B. brassicae* and the generalist *M. persicae* as well as the generalist lepidopteran *S. exigua* consistently increased transcript levels of two genes involved in early steps of aliphatic biosynthesis, *MAM1* and *CYP79F1*, and UGT, which encodes a later step (Fig. 1). *MAM1* encodes the methylthioalkylmalate synthase catalyzing the first and second cycle in methionine side-chain elongation, which results in butyl-type GS (Kroymann et al., 2001), the major type in Col WT. Accordingly, the aliphatic GS content increased significantly in these insect-treated plants compared to controls primarily due to an increase in short-chain aliphatic GS, mainly 4-methylsulfinylbutyl GS.

By contrast, after feeding by the specialist lepidopteran (*P. rapae*), no changes in aliphatic GS were observed and only a small increase in indolyl GS, especially 1-methoxyindol-3-ylmethyl GS, was documented. However, feeding by

Table 2
Insect induced changes in transcript levels of glucosinolate biosynthesis and selected defense signaling genes in *A. thaliana* Columbia wild-type and signaling mutants after one week feeding of aphids (plants 39 days old) or one day feeding of *S. exigua* (plants 38 days old)

Genes	Mean change in transcript level (assigned ranks)											
	Col WT			*coi1*			*npr1*			*etr1*		
	Aphid exp.		*Cat. exp.*	*Aphid exp.*		*Cat. Exp.*	*Aphid exp.*		*Cat. exp.*	*Aphid exp.*		*Cat. exp.*
	M. persicae	*B. brassicae*	*S. exigua*	*M. persicae*	*B. brassicae*	*S. exigua*	*M. persicae*	*B. brassicae*	*S. exigua*	*M. persicae*	*B. brassicae*	*S. exigua*
GS-Biosynthesis genes												
MAM3	+/−	+/−	++	− −	− −	− −	+/−	+/−	− −	+/−	+	+/−
MAM1	+	++	++	− −	− −	− −	+/−	+/−	+/−	−	+/−	+/−
CYP79F1	+/−	+	++	− −	− −	− −	+/−	+/−	+/−	−	+/−	+/−
CYP79B2	++	+	+	−	−	+/−	+/−	+/−	+/−	++	++	+/−
CYP79B3	+/−	+	+	+/−	+/−	+	+/−	+/−	+/−	+/−	+/−	+/−
CYP83A1	+/−	+/−	+	−	−	+	+/−	+/−	+/−	+/−	+/−	+/−
CYP83B1	+/−	+/−	+/−	−	+/−	+	+/−	+/−	+/−	+/−	+/−	+/−
UGT	+	++	++	−	+/−	+/−	−	−	+/−	++	++	+/−
Signaling pathway genes												
HEL/PR4	+/−	+/−	+	−	−	+/−	+	+/−	+/−	+/−	+/−	-
BGL1	+/−	+/−	+/−	− −	+/−	+/−	+/−	+/−	+/−	+/−	+/−	+/−
CaEF	+/−	+	++	−	−	+/−	+	+/−	+/−	+/−	+/−	+/−
PR1	+	++	++	−	−	+/−	/	/	/	+/−	+/−	+/−

The signaling mutants are blocked in jasmonate (*coi1*), salicylate (*npr1*), and ethylene (*etr1*) signaling, respectively.
++, ⩾2-fold increase; $p \leqslant 0.05$; +, 1.5–2-fold increase, $p \leqslant 0.1$; +/−, no change; −, 1.5–2-fold decrease, $p \leqslant 0.1$; − −, ⩾2-fold decrease, $p \leqslant 0.05$; /, expression too low; exp., experiment; cat; caterpillar; Calculation of significant differences in transcript levels between treatments by Kruskal–Wallis test.

P. rapae did induce the expression of genes of aliphatic GS biosynthesis (*MAM1* and *CYP83A1*, but not *CYP79F1*) and indolyl GS biosynthesis (*CYP79B2*, *CYP79B3*, and *CYP83B1*). These data suggest that GS formation in *P. rapae*-fed plants is not regulated by the transcript levels of these genes. Conceivably, a plant or insect-produced metabolite may act to decrease substrate flow into GS pathways or block biosynthesis at a later step. Our results are generally consistent with previous work, showing greater accumulation of indolyl than aliphatic GS in *Arabidopsis lyrata* L. and *Brassica oleraceae* L. upon damage by *P. rapae* (Agrawal and Kurashige, 2003). Reymond et al. (2004) showed that *P. rapae* upregulated genes of Trp biosynthesis and the subsequent Trp aldoxime formation and oxidation steps, *CYP79B2* and *CYP83B1*, as shown here but genes of aliphatic GS biosynthesis were not measured in their study. The authors further demonstrated that the genes *CYP79B2* and *CYP83B1* were not strongly induced by a related generalist, *Spodoptera littoralis* (Boisduval), which corresponds with transcript data of *S. exigua* induction (Table 1). After three days feeding of *S. exigua* on Col WT increases in aliphatic and indolyl GS were observed. This fact might indicate stronger responsiveness of the aliphatic than indolyl GS biosynthesis pathway to *S. exigua* compared to *P. rapae.*

The marked differences in plant responses to various insect herbivores may arise from differences in the biochemical composition of insect saliva (Alborn et al., 2003). Components of some caterpillar saliva have been shown to downregulate the JA signaling pathway by upregulation of the SA pathway (Felton and Korth, 2000). Oral secretions of *S. exigua* larvae contain a fatty acid-amino acid conjugate, *N*-[17-hydroxylinolenoyl]-L-glutamine (volicitin) and related hydroxyl acid and glutamine conjugates, which induce volatile emissions in corn and tobacco (Alborn et al., 2000). However, such conjugates are most likely not produced in *P. rapae* (Reymond et al., 2004), and this absence may be responsible for the differences in gene expression and GS content observed.

Phloem-feeding aphids damage plants in a completely different manner than leaf-chewing lepidopterans, and these feeding differences results in different expression levels of defense signaling genes, consistent with our current knowledge of signal transduction pathways. Transcripts of *BGL1*, induced by JA (Stotz et al., 2000) and *JMT*, upregulated after mechanical wounding as well as after methyl jasmonate treatment (Seo et al., 2001), did not change after aphid feeding in Col WT. These genes were not upregulated by *S. exigua*, but *P. rapae* induced *BGL1* and this may explain the accumulation of indolyl GS, which are known to be responsive to JA (Kliebenstein et al., 2002; Mikkelsen et al., 2003). Transcripts of *CaEF*, responsive to ET (Stotz et al., 2000), also did not change upon aphid feeding, and were only induced by the lepidopterans. Expression levels of *HEL* (*PR4*) were induced by the specialist *P. rapae* as reported by Reymond et al. (2000), but levels did not change after plant exposure to the other

herbivores. Insects of both feeding guilds induced increases in transcripts of *PR1*. The latter gene is usually expressed after pathogen infection and associated with the SA pathway (Moran and Thompson, 2001).

3.2. Jasmonate, ethylene, and salicylate signaling are all involved in glucosinolate induction after insect herbivory

We assessed the effect of insect herbivory on GS biosynthetic gene transcripts and GS accumulation in *A. thaliana* lines with compromised defense signaling pathways to get insight into which signaling cascades are most important for insect induction of GS. Blocking the perception of JA and/or ET has been documented to lead to increased susceptibility of plants to several pests and pathogens (McConn et al., 1997; Pietierse et al., 1998; Thomma et al., 1999). Mutants with compromised JA signaling, include *coi1*, which is insensitive to JA, and *cev1*, which has constitutive expression of JA and ET response (Feys et al., 1994; Ellis and Turner, 2001). Activation of JA-dependent defense genes and suppression of SA-dependent defense genes in *cev1* enhanced resistance to the aphid *M. persicae* as well as the pathogens *Erysiphe cichoracearum* (F.) and *Pseudomonas syringae* pv. *maculicola* (Ellis et al., 2002). Due to compromised JA signaling, plants of *coi1* have much lower GS content than Col WT (Figs. 2 and 3) and a lower resistance to most of the insect herbivores studied (Fig. 6), as was reported in a previous study with *M. persicae* (Ellis et al., 2002). Interestingly, most GS biosynthesis genes which are upregulated upon feeding by *M. persicae*, *B. brassicae*, and *S. exigua* in Col WT responded oppositely or not at all in *coi1*, suggesting that JA signaling is necessary for the induction of these genes. However, the situation is more complex in the case of *P. rapae*. Feeding of this specialist lepidopteran did not elicit aliphatic GS accumulation in Col WT, but did on JA-insensitive *coi1* plants. The *COI1* gene encodes an F-box protein to assemble SCF^{COI1} complexes in planta, which is assumed to regulate the abundance of substrate proteins essential for jasmonate responses (Xu et al., 2002; Xiao et al., 2004). The constitutive levels of *PR1* expression in *coi1* was higher than in Col WT (data not presented) as reported by Devadas et al. (2002), indicating that a *COI1*-dependent signal normally suppresses *PR1* in untreated plants.

The importance of JA signaling in especially indolyl GS induction was shown by Piotrowski et al. (2004). However, other signal cascades are also involved in regulating GS accumulation since *coi1* plants, though their GS levels are low, did show significant increases in aliphatic GS but not indolyl GS after feeding by *P. rapae* and *S. exigua* (Fig. 3b). For example, in the ET-insensitive mutant *etr1* the induction of aliphatic GS accumulation by aphids and *S. exigua* did not occur. Corresponding, the transcript levels of aliphatic GS biosynthetic genes, like *MAM1* and *CYP79F1*, did not increase in *etr1* as they did in Col WT (Tables 1 and 2). ET may also play a central role in the plant response to *P. rapae*, since feeding by this specialist induced the ET and JA responsive genes, *CaEF* and *BGL1*, in Col WT to a greater extent than the other herbivores tested. Within the two signaling pathways, *ERF1* (ethylene response factor 1) has been shown to be a convergence point between ET and JA pathways (review in Devoto and Turner, 2005). ET signaling may in fact suppress or modify some plant responses to insect feeding. The *etr1* line exhibited a much stronger increase in indolyl GS accumulation after *P. rapae* feeding than Col WT, highlighted by a tenfold increase of 1-methoxyindol-3-ylmethyl GS. As a consequence, *etr1* was less suitable as host plant for *P. rapae* than Col WT, when insect performance was measured as weight gain per leaf area eaten. Other examples are known in which ET signaling after herbivory suppresses some but not all plant defense responses (Kahl et al., 2000; Stotz et al., 2000).

SA-dependent signaling is known to be involved in defense responses to pathogens and aphids. Recent studies indicate that signaling, responses, and resistance to pathogens and insects partially overlap but antagonism can occur (Preston et al., 1999; Stout et al., 1999). The induction of the SA-associated signaling gene *PR1* (Moran and Thompson, 2001) by both aphids and lepidopterans in this study suggests SA involvement in the defense responses documented here. Among its many functions, SA antagonizes JA signaling (Bruxelles and Roberts, 2001) and so enhanced JA responses might be expected when SA signaling is impaired. For example, Spoel et al. (2003) reported that *A. thaliana* plants which are unable to accumulate SA produce 25-fold higher JA levels and showed enhanced expression of the JA-responsive genes, *LOX2*, *PDF1.2*, and *VSP*, in response to infection by *Pseudomonas syringae* pv. *tomato* DC3000. Consistent with these results, the *npr1* line blocked in SA signaling had constitutively increased aliphatic GS levels in our study. However, in previous work in which SA signaling was blocked at the stage of SA catabolism using *nahG* expressing *A. thaliana* plants, there was no effect on constitutive aliphatic GS accumulation (Mikkelson et al., 2003; Mewis et al., 2005) indicating a downstream interaction of SA and JA signaling pathways. Spoel et al. (2003) analyzed *npr1* and revealed that the antagonistic effect of SA on JA signaling requires the regulatory protein NPR1. Furthermore, Li et al. (2004) documented that another transcript factor, *WRKY70*, downstream of *NPR1* in the SA-dependent signaling pathway acts as an activator of SA-induced genes and as repressor of JA-responsive genes (Li et al., 2004).

The increased aliphatic GS level in *npr1* had predictable negative effects on suitability for both aphid species and the specialist caterpillar *P. rapae*, in comparison to Col WT (Fig. 6). Although *P. rapae* is a crucifer specialist suggesting a high tolerance to GS hydrolysis products, GS products can have negative effects on growth of this insect (Agrawal and Kurashige, 2003). Nevertheless, we cannot exclude that other secondary metabolites changed in signaling mutants that influenced insect performance. The generalist caterpillar *S. exigua* showed no differences in

performance on *npr1* vs. Col WT probably because it induced high aliphatic GS on Col WT that were similar to those in *npr1* (Figs. 2b and 4b). Although the *npr1* line had higher constitutive levels of aliphatic GS than Col WT, there was no induction after aphid or *S. exigua* feeding, pointing to the role of NPR1 and SA-signaling in these plant responses. Accordingly, there was no induction of genes of aliphatic GS biosynthesis after these insects fed on *npr1* plants.

4. Concluding remarks

In conclusion, insects from different feeding guilds and with different dietary specializations had different effects on indole and aliphatic GS accumulation in *A. thaliana* with sometimes changing in different ways. Alterations in GS content often correlate with changes in transcript levels of GS biosynthetic genes but this was not always the case. While JA signaling is considered to have a primary role in defense against chewing insects, the present study suggests that ET and SA signaling are involved as well depending on the nature of the herbivore, often in opposition to JA. Studies with additional signaling mutants as well as *A. thaliana* lines with altered GS control are needed to obtain a better understanding of how GS levels are regulated in response to insect herbivory.

5. Experimental

5.1. Plant material and cultivation

The following *Arabidopsis thaliana* (L.) genotypes were used: (1) Columbia-0 wild type (Col WT), (2) *coi1*, insensitive to JA due to coronatine insensitivity (Kloek et al., 2001), (3) *npr1*, SA-mediated (hypersensitive) responses blocked downstream of the pathway at NPR1 (Spoel et al., 2003), and (4) *etr1*, insensitive to ethylene, because the ethylene receptor ETR1 is disabled (Gamble et al., 1998). Seeds were vernalized and sown into 6×5 cm pots filled with sterile Metromix 200 (Scotts company, Marysville, OH, USA; contains sphagnum, peat moss, and horticultural perlite). Plants were kept in growth chambers at 22 ± 1 °C, $65 \pm 5\%$ relative humidity, at 250 μmol m^{-2} s^{-1} light intensity, and on a 10:14 (L:D) photoperiod. Approximately twice a week the plants were watered as needed and fertilized every two weeks (21-7-7, Miracle Gro).

5.2. Insect rearing

The specialist aphid *Brevicoryne brassicae* (L.) and the generalist *Myzus persicae* (Sulzer) were used as phloem feeding insects and clones of both species were maintained on pak-choi plants (*Brassica campestris* L. ssp. *chinensis* cv. Black Behi). For chewing insects we selected the specialist caterpillar *Pieris rapae* L. and the generalist *Spodoptera exigua* Hübner. Eggs of *S. exigua* were obtained from Benzon Research (Carlisle, PA). Larvae were kept on commercially available artificial *Spodoptera* diet (Bioserv, Frenchtown, NJ, USA). Eggs of *P. rapae* were purchased form the Carolina Biological Supply Service (North Carolina). The caterpillars were reared on pak-choi. Both caterpillar species were transferred to Col WT plants one day before the experiments.

5.3. Plant treatment

Col WT, *coi1*, *npr1*, and *etr1* plants were exposed in separate experiments to the two classes of feeding herbivores. During the experiments, plants with insects and controls were kept in cages of transparent mylar cylinder (5 cm diameter, 9 cm high) with fine mesh gauze (<0.01 mm mesh wide) on top. The cages maintained air exchange and allowed the insects to choose their feeding sites.

In the two aphid experiments, 10 plants per genotype were exposed to 10 *M. persicae* or *B. brassicae* (adult and 4th nymph stage) initially. Control plants and treated plants (also caged) were kept in a growth chamber at 22 ± 1 °C, with 200 μmol m^{-2} s^{-1} light intensity, and a 12:12 h photoperiod. After seven days exposure to aphids, the plants were harvested at this time they were 39 or 40 days old. In aphid treatments, the number of aphids was counted before plant harvest. Plants were cut directly above the root, immediately flash frozen in liquid nitrogen and stored at −80 °C. Plants for GS analysis were harvested in pairs (four replicate pairs) and two plants per treatment were harvested separately for molecular biological studies. RT-PCR was performed for both experiments only on Col WT.

In the caterpillar experiments, larvae were weighed and one larva was transferred to each of eight plants of each genotype. Plants with caterpillars and control plants (also caged) were kept in a growth chamber at 22 ± 1 °C, with 250 μmol m^{-2} s^{-1} light intensity, and a 10:14 h photoperiod. One experiment was conducted with *P. rapae* and *S. exigua* 2nd instar larvae, which fed for three days. At the time of harvest the plants were 42 days old. In the second experiment (Table 2), *S. exigua* larvae fed for one day on plants. *P. rapae* were not used in this experiment. Plants were harvested on the following day and were 38 days old. Before cutting the plants, the plant damage was estimated and the rosette diameter and the larval weight were measured for calculating weight increase per leaf area eaten. Plants were harvested as described for the aphid experiments (three replicate pairs). RT-PCR was performed for Col WT only in the experiment with *P. rapae* (Table 1) and was done for all genotypes in the experiment with *S. exigua* (Table 2) .

The statistical significance of variation in aphid population size on genotypes as well as weight gain per leaf area eaten was determined using analysis of variance (ANOVA) followed by the post hoc test Tukey's HSD in the program SYSTAT 10.0.

5.4. Glucosinolate analysis

GS were extracted from 20 mg lyophilized tissue in 70% boiling MeOH as described in detail in Mewis et al. (2005). 4-Hydroxybenzyl GS (The Royal Veterinary and Agricultural University, Copenhagen, Denmark) was used as internal standard for quantification of GS in extracts. GS in extracts were desulfated with 150 μl aryl sulfatase solution (Sigma–Aldrich Corp., H-1 from *Helix pomatia*) on DEAE Sephadex A-25 mini columns; column wash steps are described in Mewis et al. (2005). Desulfated extracts were analyzed by HPLC (Waters WISP 710 B) fitted with a C-18 reverse-phase column (Spherisorb ODS-2,5 μm, 4.6 mm × 250 mm, Sigma–Aldrich Corp., St. Louis, MO) with solvents (A) dd H_2O and (B) 20% CH_3CN (HPLC grade in dd H_2O) gradient at a flow rate of 1.5 ml^{-1}. The 39 min run consisted of 1% (v/v) B (1 min), 1–99% (v/v) B (20 min), 3 min hold at 99% (v/v) B, 99% to 1% (v/v) B (5 min), and a 10 min final hold at 1% (v/v) B. The eluent was monitored by photodiode array detection between 190 and 360 nm. GS peaks at 229 nm were identified using retention time and UV spectra, and GS identities were confirmed by LC-MS, Perseptive Voyager-DESTR MALDI-TOF system. For calculation of molar concentration of individual GS, relative response factors (RF) (Brown et al., 2003 and Buchner, 1987) were used to correct for absorbance difference between the reference standard (4-hydroxybenzyl GS, RF 0.51) and other compounds. Statistical differences in GS content among treatments were determined by ANOVA following by Tukey's HSD test.

5.5. Semi-quantitative RT-PCR analysis

Total RNA was isolated from frozen *A. thaliana* rosettes with the Quiagen RNeasy Plant Mini Kit in the Col WT experiment with *P. rapae* (Table 1) or with TRIzol reagent (Invitrogen, Table 2) following the standard protocols. RNA was quantified by UV spectroscopy and its integrity was visually assessed on ethidium bromide stained agarose gels. RNA was first converted to cDNA by reverse transcription according to the Promega (Madison, WI) protocol. A primer (0.5 μg), oligodT12-18 (Invitrogen, Carlsbad, CA) was added to 2 μg of total RNA with a total of 8 μl volume and was heated to 65 °C for 5 min. cDNA was synthesized by adding 0.5 mM dNTPs, 200 U of Moloney murine leukemia virus reverse transcriptase (Promega) and the buffer supplied for this enzyme in a total volume of 20 μl. The mixture was incubated at 37 °C for 1 h. The volume was adjusted to 50 μl followed by heating for 10 min at 70 °C.

The PCR reaction was optimized for each primer pair with different annealing temperatures, $MgCl_2$ concentrations, cycles, and amounts of template. After determining optimal annealing temperature and $MgCl_2$ concentration, a number of cycles was picked to insure that the PCR reaction with all primers was in the plateau phase. An aliquot (0.5 μl) of RT reaction was used as template for the tests. Then the amount of template was reduced until bands nearly disappeared to insure that the reaction was in the linear range. The following reaction condition were used for the PCR: a total volume of 20 μl, 1× PCR buffer (Promega), 0.2 mM dNTP's, 2.1 mM $MgCl_2$, 0.5 μmol of the forward and reverse primer, 1 U of *Taq* DNA polymerase (Promega), and 0.15 μl RT template. The following PCR program was performed using a RoboCycler (Stratagene): 2 min 94 °C, 30 cycles of 1 min at 94 C, 1 min 54 °C, and 1 min at 72 °C, followed by a 7 min final at 72 °C. Specific primers on the 3′ end, including UTR regions of respective genes were created and yielded products of around 200–400 bp. *Actin8* (AC8, At1g49240) was used as reference gene and was designed to be intron spanning for possible detection of DNA contamination. The *AC8* forward (f) primer was 5′-ATGAAGATTAAGGTCGTGGCAC and the reverse (r), 5′-GTTTTTATCCGAGTTTGAAGAGGC. Primers listed below were designed to amplify genes encoding enzymes for GS biosynthesis followed by genes linked to signaling pathways (the AGI codes are given in Table 1):

MAML(3)	(f) 5′-AATCACAGACGCTGATCTGA
	(r) 5′-TGACTTAATACCATCGTAGTAC-CA
MAM1	(f) 5′- AGAATCACGGATGCTGATTTG
	(r) 5′-GGAAATTGATATTACTGTGGTA-CAC
IPMS1	(f) 5′-CAAGAGTTCTTATCCGTGGAAG
	(r) 5′-AAAAGAACCTAACTTCTGTCTG-AC
IPMS2	(f) 5′-GACAACAACTACTCATCAACAA-AC
	(r) 5′-TTGATCTCTGAGATTTGCAGGTA
Cyp79F1	(f) 5′-AGTTCCGTCCTTAAGAAGAAGA
	(r) 5′-TTATTATGAAGTGACGGAGATTC
Cyp79F2	(f) 5′-GCTAAGCCTCTTCTTTTGTCTGT
	(r) 5′-TCATGATTAAGTTACGGAGATA-CA
Cyp79B2	(f) 5′-CACTTGGAAGCTACCTGAGA
	(r) 5′-GAACCAAGAAACCAACACACA
Cyp79B3	(f) 5′-TTAAGTGGAAACTAGCAGGAAG
	(r) 5′-CAAAAGGACCAAAACCGAAC
Cyp83A1	(f) 5′-GCGAACCTTCTCCTCAGC
	(r) 5′-GAGAGACGATTGCCATTTAAG
Cyp83B1	(f) 5′-TGGGATTGCAATGGTAGAGA
	(r) 5′-CATTGGTCACGCCATATCTAC
UGT	(f) 5′-GAAAGGAGTGATGGAAGGAG
	(r) 5′-TTGAATCACAGTCATCGTGGT
JMT	(f) 5′-ACCCGAAGCCCTAGCTAGT
	(r) 5′-AATCATGTCATTGTAGGCCCA
HEL	(f) 5′-GAGAATAGTGGACCAATGCAG
	(r) 5′-GTAGACCGATCGATATTGACCT
CaEF	(f) 5′-GTAACCGAGGGAAATCGAATG
	(r) 5′-TCCCCAAACTGAATAACAAGAC
BGL1	(f) 5′-CGAGGTTCGGACTTTACTACA
	(r) 5′-CGAACAACTCACAAGTCCTTC
PR1	(f) 5′-AGTCAGTGAGACTCGGATGTG
	(r) 5′-CATCCTGCATATGATGCTCCT

All primers successfully amplified a band of correct size and PCR products were cloned into the T-overhang vector pCR2.1 with the TOPO TA cloning kit (Invitrogen). PCR products were fully sequenced to confirm their fidelity. For quantification of expression profiles, the intensities of bands were visualized by gel electrophoresis on a 1.5% agarose gel containing ethidium bromide on a Kodak Image Station 2000R. Band intensities were measured and the amount calculated by using the low mass ladder (Invitrogen) and then normalized to AC8. The average gene expression levels in insect treatments for each genotype were related to their controls to estimate the changes (fold changes) in transcript levels. All RT-PCR experiments were repeated four times with two independent RT reactions from RNA obtained from same treated tissue. Excluded from further analysis were genes, such as *CYP79F2* (At1g16400, cytochrome P450 family) and *JMT* (At1g19640, jasmonic acid carboxyl methyltransferase) where no band was detected. *CYP79F2* is considered to be root specific (Chen et al., 2003), and therefore it was not surprising to find no expression in the rosette leaves analyzed. For statistical analysis of differences in gene transcript levels of treatments in Col WT, a non-parametric equivalent to analysis of variance and mean comparison, the Kruskal–Wallis test, was performed.

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