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The glutathione-deficient mutant *pad2-1* accumulates lower amounts of glucosinolates and is more susceptible to the insect herbivore *Spodoptera littoralis*

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Summary

Plants often respond to pathogen or insect attack by inducing the synthesis of toxic compounds such as phytoalexins and glucosinolates (GS). The Arabidopsis mutant *pad2-1* has reduced levels of the phytoalexin camalexin and is known for its increased susceptibility to fungal and bacterial pathogens. We found that *pad2-1* is also more susceptible to the generalist insect *Spodoptera littoralis* but not to the specialist *Pieris brassicae*. The *PAD2* gene encodes a γ-glutamylcysteine synthetase that is involved in glutathione (GSH) synthesis, and consequently the *pad2-1* mutant contains about 20% of the GSH found in wild-type plants. Lower GSH levels of *pad2-1* were correlated with reduced accumulation of the two major indole and aliphatic GSs of Arabidopsis, indolyl-3-methyl-GS and 4-methylsulfinylbutyl-GS, in response to insect feeding. This effect was specific to GSH, was not complemented by treatment of *pad2-1* with the strong reducing agent dithiothreitol, and was not observed with the ascorbate-deficient mutant *vtc1-1*. In contrast to the jasmonate-insensitive mutant *coi1-1*, expression of insect-regulated and GS biosynthesis genes was not affected in *pad2-1*. Our data suggest a crucial role for GSH in GS biosynthesis and insect resistance.

Keywords: glutathione, glucosinolates, *Spodoptera littoralis, Pieris brassicae,* defence gene expression, ascorbate.

Introduction

Plants have evolved sophisticated strategies to cope with attack by herbivorous insects, including constitutive and inducible defences. Induced defences are either indirect, manifested by the release of volatiles to attract predators of chewing larvae (Dicke *et al.*, 2003), or direct, as illustrated by the synthesis of anti-digestive proteins, toxic secondary compounds, proteases or lectins (Karban and Baldwin, 1997; Walling, 2000). Insect herbivory is known to activate defence responses in plants, including crops such as tomato (Howe *et al.*, 1996), potato (Johnson *et al.*, 1989; Ryan, 1990), maize (Pechan *et al.*, 2002) or poplar (Constabel *et al.*, 2000). Global transcript profiling has led to the identification of numerous genes that are differentially expressed after insect attack (Korth, 2003; Reymond *et al.*, 2000; Voelckel and Baldwin, 2004). Experiments with chewing insects, cell content-feeding thrips and phloem-feeding aphids identified major expression changes specific to each attacker, underlying the complexity and specificity of transcriptional changes after insect attack in Arabidopsis (De Vos *et al.*, 2005; Reymond *et al.*, 2004). In contrast, comparison of Arabidopsis responses to a specialist and a generalist insect revealed strikingly similar transcript profiles (Reymond *et al.*, 2004).

A network of inter-dependent signal transduction pathways controls the outcome of plant–pathogen interactions, and includes the main regulators jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) (Glazebrook, 2001; Katagiri, 2004; Pieterse and van Loon, 1999; Reymond and Farmer, 1998). JA has been shown to be crucial for defence against insects (Howe *et al.*, 1996; Kessler *et al.*, 2004; McConn *et al.*, 1997; Reymond *et al.*, 2004). For example, Arabidopsis *coronatine-insensitive 1* (*coi1-1*) mutants

impaired in the perception of JA have lost the ability to induce the majority of genes regulated by larval feeding of two lepidopteran species, *Pieris rapae* and *Spodoptera littoralis*, and consequently are more susceptible to these insects (Bodenhausen and Reymond, 2007; Reymond *et al.*, 2004).

Plant secondary metabolites play an important role in defence, as many of them are toxic to pathogens and insects. In Brassicaceae, sulfur-containing indole derivatives such as camalexin in Arabidopsis act as phytoalexins, with antimicrobial and anti-fungal properties (Glazebrook, 2005). However, whether camalexin is involved in defence against insects is unknown. Glucosinolates (GS) represent another class of sulfur-containing compounds characteristic of the Brassicaceae. Upon tissue damage, GS react with myrosinase enzymes stored in specialized cells. The resulting hydrolysis produces toxic thiocyanates, isothiocyanates or nitriles, which are thought to play an important role in defence against herbivores (Rask *et al.*, 2000). GS are grouped into aliphatic, aromatic and indole GS depending on the origin of the amino acid-derived side chain (Wittstock and Halkier, 2002). Like camalexin, indole GS are derived from the amino acid precursor tryptophan.

GS form a constitutive defence (Wittstock and Gershenzon, 2002), but there is also evidence that these compounds accumulate in response to elicitors, JA treatment, wounding or herbivory (Bartlet *et al.*, 1999; Brader *et al.*, 2001; Doughty *et al.*, 1995; Mikkelsen *et al.*, 2003). A positive correlation between increased levels of aliphatic GS and reduced performance of generalist herbivores was demonstrated in Arabidopsis (Kliebenstein *et al.*, 2005; Mewis *et al.*, 2005), but this was not observed for the specialist *Plutella xylostella* (Kliebenstein *et al.*, 2005). Specialist herbivores are predicted to be less affected by chemical defences compared to generalists, although the finding that survival and development of larvae of the specialist *P. rapae* were reduced when allyl isothiocyanate, a breakdown product of GS, was incorporated in the diet (Agrawal and Kurashige, 2003) has recently challenged this view. Arabidopsis mutants with altered GS metabolism have been used to test the role of GS on the performance of herbivores. Over-expression of the R2R3–MYB transcription factor *MYB51* led to increased accumulation of indole GS and reduced insect herbivory by *Spodoptera exigua* (Gigolashvili *et al.*, 2007). In line with these findings, overexpression of a positive regulator of GS accumulation, identified as a calmodulin-binding protein (IQD1), reduced insect herbivory by *Trichoplusia ni* and decreased infestation by the green peach aphid *Myzus persicae* (Levy *et al.*, 2005). Moreover, it was shown that a mutant lacking two major myrosinase genes, *TGG1* and *TGG2*, was more susceptible to the generalist *Trichoplusia ni* and the facultative Solanaceae specialist *Manduca sexta*, but was more resistant to the specialist *P. rapae* (Barth and Jander, 2006).

In this study, we analysed the response of the phytoalexin-deficient Arabidopsis mutant *pad2-1* to insect herbivory. This mutant has a higher susceptibility to virulent strains of *Pseudomonas syringae*, to the oomycete pathogen *Phytophtora brassicae*, and to some fungal pathogens (Glazebrook and Ausubel, 1994; Parisy *et al.*, 2007; Roetschi *et al.*, 2001). The *PAD2* gene was recently shown to encode a γ-glutamylcysteine synthetase (Parisy *et al.*, 2007), the enzyme that catalyses the first committed step of glutathione (GSH) synthesis. Consequently, *pad2-1* was shown to contain about 20% of the wild-type amounts of GSH, suggesting an important role for cellular redox homeostasis in secondary metabolite production and disease resistance (Parisy *et al.*, 2007). As herbivory triggers the up-regulation of several genes involved in the antioxidant defence system (Reymond *et al.*, 2004), our goal was to evaluate the implications of GSH deficiency on insect resistance. We found that reduced GSH biosynthesis leads to increased plant susceptibility to the generalist insect *S. littoralis* without modifying the expression of insect-regulated genes. This enhanced susceptibility was not due to camalexin deficiency, was specifically due to the lower GSH levels, and was correlated with reduced accumulation of GS in *pad2-1* plants.

Results

The pad2-1 *mutant is more susceptible to insect attack*

The *pad2-1* mutant contains about five times less GSH than wild-type plants and is more susceptible to several fungal, oomycete and bacterial pathogens (Glazebrook and Ausubel, 1994; Parisy *et al.*, 2007; Roetschi *et al.*, 2001). To determine whether this mutant is also affected in its resistance to herbivory, *pad2-1* plants were challenged with larvae of the generalist insect *S. littoralis*. Freshly hatched larvae were placed on mutant and wild-type plants and were weighed after 8 days of feeding. We found that *pad2-1* plants experienced more leaf damage than wild-type plants (Figure 1a). In accordance with this observation, larvae feeding on *pad2-1* were 2.4 times heavier than larvae feeding on Col-0 (Figure 1b). This experiment was independently repeated four times, and larvae always gained more weight when feeding on *pad2-1* (2.35 ± 0.3-fold, $n = 4$).

To verify that the increased susceptibility of *pad2-1* to *S. littoralis* was indeed linked to the reduced activity of GSH1, two complemented 35S::*GSH1 pad2-1* lines with wild-type GSH content (Parisy *et al.*, 2007) were challenged with *S. littoralis* larvae, and it was found that larvae were not significantly heavier than when feeding on wild-type plants, suggesting that the reduced GSH level was indeed responsible for the observed phenotype (Figure 1b).

Glucosinolates (GS) represent an important constitutive chemical defence against herbivores in the Brassicaceae

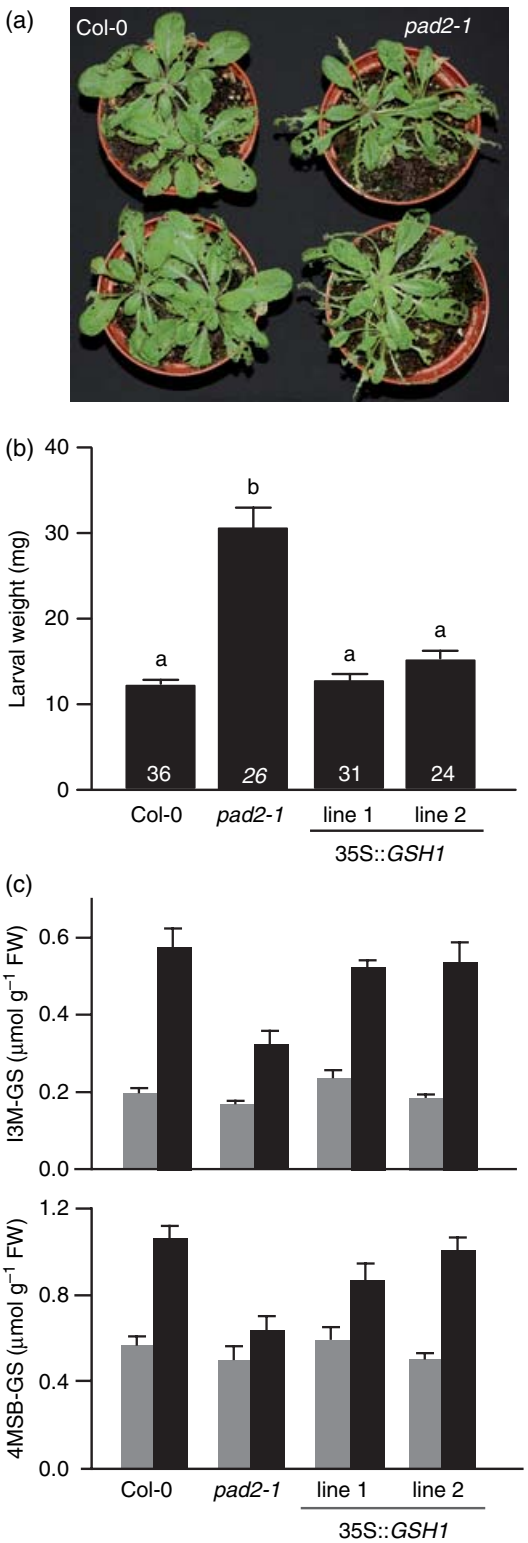

Figure 1. Herbivore resistance and glucosinolate accumulation in glutathione-deficient *pad2-1* plants in response to *Spodopotera littoralis*.
(a) Effect of feeding by *S. littoralis* on Col-0 and *pad2-1* plants. Three freshly hatched *S. littoralis* larvae were placed on each pot, and photographs were taken after 8 days of feeding. Col-0 plants (left pots) clearly show less damage than *pad2-1* plants (right pots).
(b) Larval weight measured after feeding on Col-0, *pad2-1* and complemented lines of *pad2-1* (35S::*GSH1* lines). Freshly hatched *S. littoralis* larvae were placed simultaneously on each Arabidopsis genotype, and larval weight (mean ± SE) was measured after 8 days of feeding. Bars with different letters differ at $P < 0.05$ (Tukey's HSD test). The number of larvae used in each experiment is shown within the bars.
(c) Levels of the most abundant indole GS (indolyl-3-methyl-GS, I3M-GS) and the most abundant aliphatic GS (4-methylsulfinylbutyl-GS, 4MSB-GS) were quantified in control plants (grey bars) and in plants challenged with *S. littoralis* (black bars). Thirty-day-old Col-0, *pad2-1* and two complemented lines of *pad2-1* (35S::*GSH1* lines) were analysed. Complemented lines 1 ($P = 0.10$) and 2 ($P = 0.69$) did not show a reduced induction of I3M-GS compared with Col-0, but *pad2-1* ($P < 0.05$) did show a reduced induction. Similarly, complemented lines 1 ($P = 0.14$) and ($P = 0.87$) did not show a reduced induction of 4MSB-GS compared with Col-0, but *pad2-1* ($P < 0.05$) did show a reduced induction (full-factorial ANOVA, genotype × treatment interaction effect). Values (±SE) are the mean of four (I3M-GS) or three (4MSB-GS) independent measurements.

family. Herbivory, JA treatment and mechanical wounding have been found to induce indole GS levels in Arabidopsis (Mikkelsen *et al.*, 2003). As GS are sulfur-containing molecules, we hypothesized that GSH deficiency might interfere with the accumulation of these metabolites. The levels of indolyl-3-methyl-glucosinolate (I3M-GS) and 4-methylsulfinylbutyl-GS (4MSB-GS), which make up 70% of the total GS in Col-0 leaves (Kliebenstein *et al.*, 2001), were enhanced 3- and 1.6-fold, respectively, after *S. littoralis* feeding in wild-type plants. In contrast, induction of I3M-GS and 4MSB-GS in response to insect feeding was much reduced in *pad2-1* (Figure 1c). In accordance with the feeding experiments, the two complemented 35S::*GSH1 pad2-1* lines showed wild-type accumulation of I3M-GS and 4MSB-GS in response to *S. littoralis*, indicating that the reduced GSH level of *pad2-1* was responsible for the altered GS profile (Figure 1c).

GS contribute to resistance against the generalist S. littoralis

Arabidopsis mutants affected in the biosynthesis of indole GS or aliphatic GS were used to determine the individual contributions of these GS classes to the resistance against the generalist herbivore *S. littoralis*. The Arabidopsis double mutant *cyp79B2 cyp79B3* (Zhao *et al.*, 2002) is impaired in two cytochrome P450 enzymes, encoded by *CYP79B2* and *CYP79B3*, that are implicated in the specific conversion of tryptophan to indole-3-acetaldoxime, the precursor of indole GS (Hull *et al.*, 2000; Mikkelsen *et al.*, 2000). Indole GS, including the most abundant I3M-GS, are not present at detectable levels in the *cyp79B2 cyp79B3* double mutant, whereas aliphatic GS levels are normal (Zhao *et al.*, 2002). Larval weight increased 1.7-fold on *cyp79B2 cyp79B3* compared to Col-0, demonstrating that indole GS play a role as defensive compounds against *S. littoralis* but contribute to only part of the COI1-dependent defence response, as larval weight increased 5.2-fold on *coi1-1* (Figure 2a). This

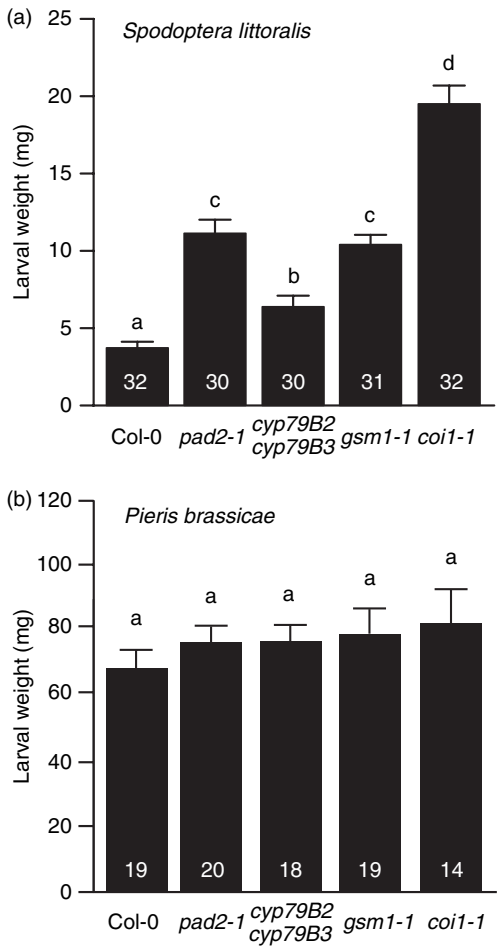

Figure 2. Performance of a generalist and a specialist insect on indole and aliphatic glucosinolate mutants.
Larval performance was tested on *pad2-1*, on the *cyp79B2 cyp79B3* double mutant, which lacks indole GS, and on the *gsm1-1* mutant, which lacks several abundant aliphatic GS. Col-0 and *coi1-1* plants were used as references. Freshly hatched larvae of the generalist *Spodopotera littoralis* (a) and the specialist *Pieris brassicae* (b) were placed simultaneously on each Arabidopsis genotype, and larval weight (mean ± SE) was measured after 8 days of feeding. Bars with different letters differ at $P < 0.05$ (Tukey's HSD test). The number of larvae used in each experiment is shown within the bars.

experiment was repeated four times, and on average larvae were 1.5 ± 0.07-fold heavier ($n = 4$) when feeding on the double mutant.

We next evaluated the importance of aliphatic GS in resistance to herbivory. The *gsm1-1* mutant has about 60-fold less 4MSB-GS, and about 50-fold less 4-methylthiobutyl-GS (Haughn *et al.*, 1991; Kroymann *et al.*, 2001). The levels of other aliphatic GS are less reduced in *gsm1-1*, and the shorter chain aliphatic 3-methylsulfinyl-GS is increased 3.5-fold. Moreover, indole GS are present at near wild-type levels in this mutant (Haughn *et al.*, 1991; Kroymann *et al.*, 2001). The *gsm1-1* plant carries a mutation in the gene methylthioalkylmalate synthase 1 (*MAM1*), which controls the initial reaction in elongation of the methionine chain of aliphatic GS (Kroymann *et al.*, 2001). Similarly to the double mutant impaired in indole GS biosynthesis, *gsm1-1* plants allowed a 2.8-fold greater growth of *S. littoralis* larvae compared to Col-0 (Figure 2a). In four independent experiments, larval weight increased 1.8 ± 0.4-fold ($n = 4$) on *gsm1-1* plants, suggesting that, in addition to indole GS, some aliphatic GS also contribute to defence against feeding insects. On average, both GS mutants had a susceptibility to *S. littoralis* feeding that was similar to that of *pad2-1*.

Specialist herbivores are predicted to be more resistant to plant defences compared with generalists (Bernays, 1998). We tested the susceptibility of *pad2-1* plants to larvae of the specialist *Pieris brassicae*, and compared it to that of GS mutants and *coi1-1*. Larvae grew equally well on wild-type and mutant plants, with no significant difference between the genotypes (Figure 2b). This experiment was repeated three times with *pad2-1* and *coi1-1*. A small but significantly enhanced susceptibility of *coi1-1* plants could be measured in two experiments, while *pad2-1* was slightly more susceptible than Col-0 in one experiment (data not shown). These data show that the specialist *P. brassicae* grows similarly on plants containing or lacking GS, and reinforce the hypothesis that *pad2-1* plants are more susceptible to the generalist *S. littoralis* because they accumulate less GS.

The pad2-1 *mutant shows wild-type defence gene expression in response to* S. littoralis

A dedicated DNA microarray containing insect- and defence-regulated genes (Bodenhausen and Reymond, 2007) was used to determine the effect of GSH deficiency on expression changes in *pad2-1*. Transcript signatures of *pad2-1* and wild-type plants were analysed after 8 days of feeding by *S. littoralis* larvae. Herbivory caused the induction of 189 transcripts in both genotypes, and there was almost no difference in gene expression between the two genotypes (Figure 3a). Transcripts of only one gene, a cysteine proteinase (At4g11320), accumulated to a higher level in wild-type plants, although it was still up-regulated in *pad2-1* in response to *S. littoralis* (Figure 3a and Tables S1 and S2). Surprisingly, given the reduced GS accumulation in *pad2-1* plants after herbivory, genes involved in GS metabolism were equally up-regulated in both Col-0 and *pad2-1* plants after *S. littoralis* feeding (Table 1).

In sharp contrast, the *coi1-1* mutation affected the expression of 54% of the insect-regulated genes, including the GS biosynthesis genes (Figure 3b and Table 1). This confirms that jasmonate insensitivity negatively affects GS accumulation and herbivore resistance via reduced expression of many insect-induced genes (Bodenhausen and Reymond, 2007; Reymond *et al.*, 2004).

To validate the microarray results, the expression of genes involved in the biosynthesis or breakdown of GS was tested by quantitative real-time PCR. The indole GS biosynthesis

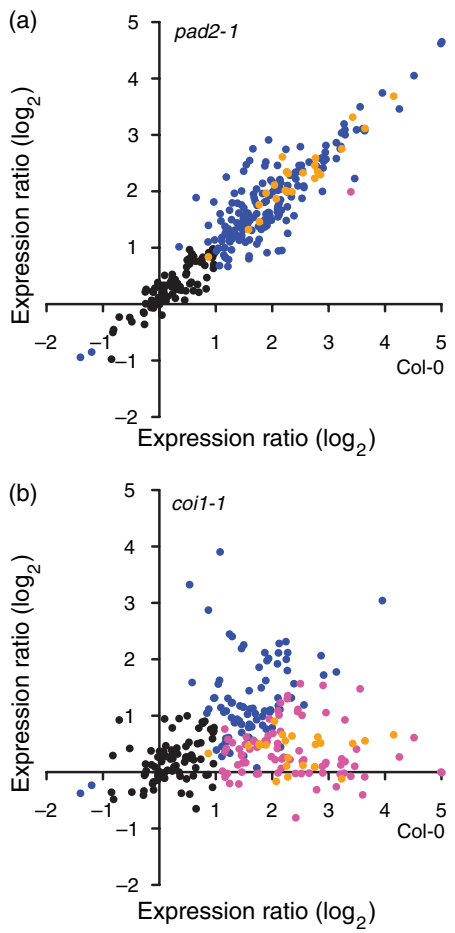

Figure 3. Comparison of expression changes in wild-type and mutant plants challenged with *Spodoptera littoralis* larvae.
Relative changes in gene expression were measured after 8 days of feeding using a dedicated microarray containing insect-regulated genes. Mean expression ratios calculated from experiments comparing challenged and unchallenged Col-0 plants are plotted against mean expression ratios between challenged and unchallenged *pad2-1* (a) or *coi1-1* (b) plants. Data are the average of five biologically independent replicates for Col-0, three for *pad2-1* and four for *coi1-1*. Black dots represent genes that showed no changes in gene expression, blue dots represent genes that were significantly induced in Col-0 and the mutant but that showed no statistical difference between genotypes, and magenta dots represent genes that were significantly different between Col-0 or mutant plants (Student's *t* test). Orange dots represent genes involved in GS biosynthesis (see Table 1).

genes *ASA1* (At5g05730), *CYP83B1* (At4g31500), *ST5a* (At1g74100) and a gene encoding a putative myrosinase-binding protein (*MBP*, At3g16420) were all strongly induced after feeding by *S. littoralis* (Figure S1). The induction of indole GS biosynthesis genes is consistent with the observed insect-induced accumulation of I3M-GS (Figure 1c). We found that these genes were similarly induced in Col-0 and *pad2-1*. For the two genes *CYP79F1* (At1g16410) and *CYP83A1* (At4g13770), which are specifically involved in the biosynthesis of aliphatic GS, no or only a weak induction was observed in response to herbivory (data not shown).

Specific role of GSH in resistance to herbivory

Having found that *pad2-1* plants have a lower accumulation of GS in response to herbivory and show no major changes in insect-inducible gene expression, we further explored the link between lower GSH levels and the susceptibility to *S. littoralis*. As *pad2-1* is deficient in the phytoalexin camalexin, there was the possibility that this anti-microbial compound could also be effective against insects. The *pad3-1* mutant was selected to test whether reduced camalexin level could account for the enhanced susceptibility to *S. littoralis*. The *pad3-1* mutant is impaired in a cytochrome P450 (CYP71B15) that catalyses the last step of camalexin biosynthesis (Schuhegger *et al.*, 2006) and consequently lacks camalexin (Glazebrook and Ausubel, 1994). This mutant was not more susceptible to *S. littoralis* larvae, as larvae did not gain more weight on *pad3-1* than on Col-0 plants (Figure 4). This provides genetic evidence that camalexin is not important in the resistance to chewing herbivores, and that the enhanced susceptibility of *pad2-1* plants is due to other cellular changes.

GSH deficiency could negatively affect plant resistance to herbivores via disturbance of antioxidant defence and deregulation of the ascorbate–GSH cycle. The ascorbate-deficient mutant *vtc1-1*, which contains 25% of the wild-type ascorbic acid level (Conklin *et al.*, 1999), was chosen to test the effect of disturbed redox balance on insect performance. *S. littoralis* larvae gained significantly more weight on *vtc1-1* (twofold more weight in two independent experiments) than on wild-type plants, an increase that was similar to that observed with *pad2-1* plants (Figure 4). However, whereas *pad2-1* showed reduced accumulation of the indole I3M-GS following herbivory compared to Col-0, *vtc1-1* showed a wild-type profile of I3M-GS accumulation (Figure 5a). We also analysed the most abundant aliphatic GS, 4MSB-GS, and found that its levels showed similar induction in Col-0 and *vtc1-1* after herbivory, although it was less pronounced than with I3M-GS (Figure 5b). As with I3M-GS, 4MSB-GS levels were not induced by *S. littoralis* feeding in *pad2-1* (Figure 5b). We also found that herbivory induced GS biosynthesis genes similarly in Col-0 and *vtc1-1* (Figure S1). Hence, the enhanced susceptibility of *vtc1-1* to *S. littoralis* does not appear to be caused by a deficiency in GS accumulation.

The lower GSH levels in *pad2-1* plants affect the reducing potential in the cytosol, which could in turn interfere with the insect-induced accumulation of GS. To address the specificity of the GSH effect, we measured total GS levels in *pad2-1* plants in response to herbivory, and tested the effect of supplementation with GSH or another reducing agent dithiothreitol (DTT). Addition of 1 mM GSH to *pad2-1* leaves challenged with *S. littoralis* restored GS levels to those observed in challenged Col-0 leaves, whereas 1 mM DTT had no effect (Figure 6). We verified that both compounds

Table 1 Expression of genes involved in glucosinolate metabolism in response to *Spodoptera littoralis* feeding

AGI code	Description	Col-0[a]	*P* value[b]	*pad2-1*[a]	*P* value[b]	*coi1-1*[a]	*P* value[b]
At5g05730	Anthranilate synthase, alpha subunit (ASA1)	**4.78**	<0.001	**5.09**	<0.001	1.08	0.440
At1g25220	Anthranilate synthase, beta subunit (ASB)	1.83	0.005	1.79	0.001	1.26	0.004
At3g54640	Tryptophan synthase, alpha subunit (TSA1)	**4.53**	0.003	**6.11**	<0.001	1.59	0.026
At4g39950	Cytochrome P450 (CYP79B2)	**4.86**	<0.001	**4.98**	<0.001	1.19	0.036
At4g31500	Cytochrome P450 (CYP83B1)	**3.71**	<0.001	**3.91**	<0.001	1.40	0.065
At1g74100	GS sulfotransferase (ST5a)	**2.99**	<0.001	**2.50**	0.002	1.38	0.062
At1g54010	Myrosinase-associated protein	**4.85**	0.017	**3.99**	0.042	0.94	0.2865
At3g16420	Myrosinase-binding protein, putative	**10.75**	<0.001	**9.95**	<0.001	1.42	0.107
At2g33070	Lectin, similar to myrosinase binding protein	**4.20**	0.002	**3.65**	<0.001	0.89	0.286
At3g16400	Myrosinase-binding protein-like (MLP-470)	**17.79**	<0.001	**12.88**	<0.001	1.58	0.508

[a]Mean expression ratios calculated from five (Col-0), three (*pad2-1*) or four (*coi1-1)* biologically independent experiments. Values in bold have an expression ratio ≥2.
[b]*P* values calculated on $\log_2$-transformed ratios from experiments comparing Col-0 with mutant plants (Student's *t*-test).

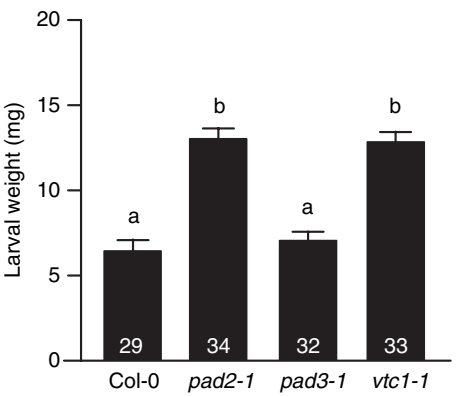

Figure 4. Influence of camalexin and ascorbate levels on larval performance of *Spodoptera littoralis*.
Larval weight was measured after feeding on Col-0, *pad2-1*, and mutants with reduced camalexin levels (*pad3-1*) or ascorbate levels (*vtc1-1*). Freshly hatched *S. littoralis* larvae were placed simultaneously on each Arabidopsis genotype, and larval weight (mean ± SE) was measured after 8 days of feeding. Bars with different letters differ at $P < 0.05$ (Tukey's HSD test). The number of larvae used in each experiment is shown within the bars. Similar results were observed in independent replicate experiments (data not shown).

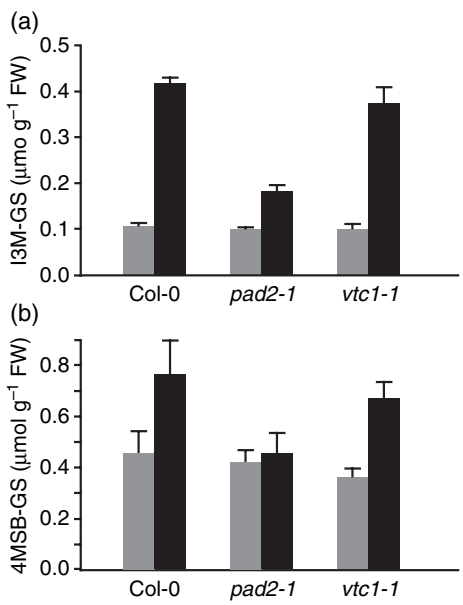

Figure 5. Glucosinolate levels in wild-type and mutant plants in response to herbivory.
(a) Levels of indolyl-3-methyl-GS (I3M-GS) were quantified in 26-day-old control plants (grey bars) and in plants challenged with *Spodoptera littoralis* (black bars). The *pad2-1* plants ($P < 0.001$) but not the *vtc1-1* plants ($P = 0.31$) showed a reduced induction of I3M compared with Col-0 (full-factorial ANOVA, genotype × treatment interaction effect).
(b) Levels of 4-methylsulfinylbutyl-GS (4MSB-GS) were quantified in 26-day-old control plants (grey bars) and in plants challenged with *Spodoptera littoralis* (black bars). The *pad2-1* plants ($P < 0.05$) but not the *vtc1-1* plants ($P = 0.981$) showed a reduced induction of 4MSB compared with Col-0 (full-factorial ANOVA, genotype × treatment interaction effect).

entered the leaves and were active by observing a similar induction of *PR1* gene expression (data not shown). The results are thus in agreement with a specific role of GSH in GS metabolism.

GSH levels decrease in wild-type plants in response to herbivory

The enhanced susceptibility of *pad2-1* to an insect herbivore suggested an important role of GSH in herbivore resistance. To test the effect of herbivory on GSH metabolism, cysteine and GSH levels were quantified in Col-0 and *pad2-1* plants after feeding by *S. littoralis*. The amount of the GSH biosynthesis substrate cysteine was higher in *pad2-1* than in Col-0, and its content significantly increased in the mutant and to a lesser extent in wild-type plants in response to herbivory (Figure 7a). The increased cysteine level of infested plants suggests a stimulatory role of herbivory on sulfur metabolism. Insect feeding caused an average 22% decrease of the GSH pool in Col-0 in four independent experiments, suggesting that GSH is increasingly metabo-

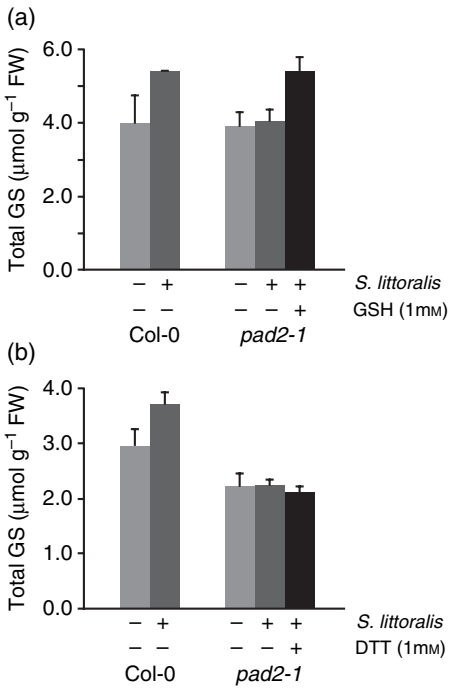

Figure 6. Physiological complementation of glucosinolate deficiency in *pad2-1* by feeding of reducing compounds.
Total GS were quantified in excised leaves of plants challenged with *Spodoptera littoralis* for 48 h in the presence or absence of GSH (a) or DTT (b). Six-week-old (a) and 5-week-old (b) control plants without herbivores (light grey bars), insect-challenged plants (dark grey bars) and insect-challenged plants supplemented with GSH or DTT (black bars) were used. Values (±SE) are the mean of three independent measurements. The experiment was performed twice with similar results. GS levels were not statistically different ($P = 0.49$, Student's *t* test) between insect-challenged Col-0 and insect-challenged *pad2-1* plants complemented with GSH, but they were statistically different between insect-challenged Col-0 and insect-challenged *pad2-1* plants complemented with DTT ($P < 0.01$, Student's *t* test).

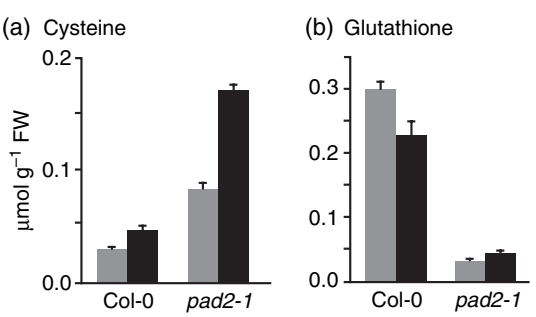

Figure 7. Effect of *Spodoptera littoralis* feeding on foliar cysteine and glutathione content.
The cysteine (a) and GSH (b) content were measured in Col-0 and *pad2-1* plants 48 h after the onset of *Spodoptera littoralis* feeding (black bars). Unchallenged plants were used as controls (grey bars). Full-factorial ANOVA indicates that the accumulation of cysteine in insect-challenged Col-0 ($P < 0.01$) and *pad2-1* ($P < 0.0001$) is significant (treatment effect for each genotype), and that *pad2-1* accumulates cysteine significantly differently than Col-0 ($P < 0.001$, genotype × treatment interaction effect). The decrease in GSH level upon herbivory in Col-0 is significant, with $P < 0.01$ (ANOVA, treatment effect). Values (±SE) are the mean of eight independent measurements. Similar results were found in four independent replicates.

lized in attacked leaves. The low GSH level of *pad2-1* remained unchanged (Figure 7b).

Is GSH a sulfur donor for GS biosynthesis?

GS and camalexin are sulfur-containing molecules. The sulfur donor has been proposed to be cysteine or a cysteine derivative, Cys-R (Halkier and Gershenzon, 2006; Mikkelsen *et al.*, 2004; Zook and Hammerschmidt, 1997), although direct experimental proof is lacking. Our observation that lower GS levels in *pad2-1* seem to be specifically due to a lower level of GSH raised the possibility that GSH (and not cysteine) directly serves as the sulfur donor in GS biosynthesis. We thus performed two sets of labelling experiments with either ^{35}S-labelled GSH (GS*H) or ^{35}S-labelled cysteine (Cys*). In the first experiment, GS*H was applied through the cut petiole of Col-0 leaves that had been challenged for 24 h with *S. littoralis*. Incorporation of radioactivity in thiols and GS was measured after 24 h of incubation. In support of our hypothesis, labelled GS were readily detected after applying GS*H to insect-challenged leaves. About 19% of the total applied radioactivity was incorporated into GS, of which 8% was found in I3M-GS and 4MSB-GS (Table S3). However, the results were inconclusive because radiolabel was also detected in the cysteine pool, which had a higher specific activity than the GSH pool.

The Cys* feeding experiments were performed in the presence of the glutathione synthetase inhibitor buthionine sulfoximine (BSO) to prevent the passage of label into the GSH pool. Leaves were challenged with *S. littoralis* for 48 h and then incubated for 4 h in treatment solution before application of Cys* through the cut petiole. Incorporation of radioactivity was measured after 24 h. Cys* feeding led to the accumulation of 25% of the total applied radioactivity in the GS pool, including 11% in I3M-GS and 4MSB-GS (Table S4). BSO treatment did not entirely prevent labelling of the GSH pool, as 14% of label was still incorporated into GS (Table S4). As BSO did not entirely prevent labelling of the GSH pool, this experiment does not allow clear identification of the origin of the radioactivity incorporated into the GS. In addition, BSO treatment caused a fourfold increase in the cysteine pool, resulting in a dilution of specific radioactivity.

Myrosinase activity is not changed in pad2-1

As the effect of GS on insects depends on the activity of myrosinase, the *pad2-1* phenotype might also be explained by a difference in myrosinase activity. We thus measured myrosinase activity in Col-0, *pad2-1* and *vtc1-1* plant extracts under physiological conditions. The rate of exogenously added sinigrin degradation was highly similar between Col-0 and *pad2-1* lines, whereas *vtc1-1* showed a much reduced myrosinase activity. This lower activity in

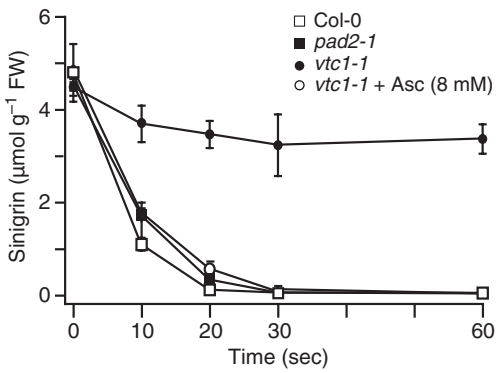

Figure 8. Myrosinase activity in Col-0, *pad2-1* and *vtc1-1* plants.
Myrosinase activity was measured by testing the rate of sinigrin degradation in extracts of different genotypes. Plants extracts were depleted of endogenous GS before the addition of exogenous sinigrin. At the indicated time points, the reaction was stopped and the remaining sinigrin was quantified by HPLC. To complement the ascorbate deficiency of *vtc1-1* mutant, ascorbate (Asc) was added with sinigrin to the plant extract. Values (±SE) are the mean of three measurements. Similar results were found in three independent replicates.

vtc1-1 could be restored to the wild-type level by addition of a physiological concentration of ascorbate to the reaction medium (Figure 8). Moreover, myrosinase activity in the jasmonate-insensitive mutant *coi1-35* was similar to that in Col-0 (data nor shown). These results indicate that the susceptibility of *pad2-1* to *S. littoralis* is not due to a lower myrosinase activity. However, the susceptibility of *vtc1-1* is probably due to the observed reduced myrosinase activity.

Discussion

This study shows that the GSH deficiency of the Arabidopsis mutant *pad2-1* causes increased susceptibility to the generalist herbivore *S. littoralis*. The enhanced susceptibility correlates with a reduced accumulation of the major Arabidopsis indole GS, I3M-GS, and the major aliphatic GS, 4MSB-GS. In addition, mutants impaired in the biosynthesis of indole or aliphatic GS were found to be more susceptible to *S. littoralis*. It is thus tempting to speculate that the reduced GS accumulation in *pad2-1* is responsible for the observed phenotype. In support of this hypothesis, we found that larval growth of the specialist insect *P. brassicae* was not different between wild-type, *pad2-1* and GS biosynthesis mutants. It has been reported that larvae of the specialists *Pieris rapae* and *P. brassicae* can overcome the GS–myrosinase defence system because they contain a nitrile-specifier gut protein that renders GS breakdown products less toxic (Wittstock *et al.*, 2004). Interestingly, this enzyme activity was absent in *S. littoralis* (Wheat *et al.*, 2007).

Because the expression of genes involved in GS biosynthesis was not changed in *pad2-1*, the effect of the PAD2 mutation on GS accumulation is not caused, as in *coi1-1*, by alterations in the transcriptional regulation of GS biosynthesis. We also showed that myrosinase activity was not affected in *pad2-1*, suggesting that the breakdown of GS was not altered in the mutant. The GSH deficiency of *pad2-1* suggests that GS synthesis is negatively affected either by a disturbance of redox homeostasis negatively affecting the activity of GS biosynthetic enzymes or by the lack of GSH as a putative sulfur donor in GS biosynthesis. Because ascorbate is an important molecule in the control of cellular redox balance, the ascorbate-deficient *vtc1-1* mutant was used to test the first hypothesis. The *vtc1-1* plants showed enhanced susceptibility to *S. littoralis*, similar to *pad2-1*. In contrast to *pad2-1*, however, *vtc1-1* showed normal accumulation of GS after herbivory, indicating that the enhanced susceptibility of *vtc1-1* is not linked to GS deficiency. The susceptibility of *vtc1-1* was correlated with reduced myrosinase activity, and this effect could be complemented by the addition of ascorbate. Ascorbate has been shown to enhance the activity of myrosinase by acting as a co-factor (Bones and Rossiter, 2006; Burmeister *et al.*, 2000). Our finding that the activity of myrosinase is much reduced in a mutant that lacks ascorbate but is normal in a mutant that has low GSH levels supports a previous report indicating that myrosinase activation requires ascorbate but that this is independent of the reducing property of this co-factor (Ettlinger *et al.*, 1961).

We showed that the reduced accumulation of GS in *pad2-1* following herbivory could be complemented by supplying GSH to the mutant, whereas the addition of another reducing agent DTT had no effect. In line with the *vtc1-1* data, this result rules out a non-specific disturbance of the redox potential as an explanation for the lower GS levels in *pad2-1*, and reinforces a specific role for GSH in the observed phenotype. Another possibility is that GSH deficiency could directly affect the biosynthesis of GS in *pad2-1*. GS and camalexin are sulfur-containing indole-derived molecules. The sulfur donor has been proposed to be cysteine or a cysteine derivative, Cys-R (Halkier and Gershenzon, 2006; Mikkelsen *et al.*, 2004; Zook and Hammerschmidt, 1997). The increased cysteine levels in plants challenged with *S. littoralis* indicate that cysteine metabolism is stimulated upon herbivory. The strongly enhanced accumulation of cysteine in *pad2-1* argues against cysteine availability being the limiting factor for GS biosynthesis. Interestingly, *S. littoralis* feeding on wild-type plants caused the GSH level to drop by about 20% compared to uninfested control plants. This increased metabolism of GSH coincides with the increased accumulation of GS upon insect feeding, suggesting that GSH could be the sulfur donor required for the formation of GS. In support of this hypothesis, it has been shown *in vitro* that non-enzymatic formation of an adduct between a nucleophile and an aci-nitro compound, which leads to *S*-alkyl-thiohydroximate, can occur with cellular nucleophilic substrates such as glutathione, cyste-

ine and 1-thio-β-D-glucose (Bak and Feyereisen, 2001; Bak *et al.*, 2001). In addition, feeding GSH to excised leaves of *pad2-1* inoculated with *Pseudomonas syringae* pv. *maculicola* restored wild-type accumulation of camalexin (Parisy *et al.*, 2007). As both glucosinolates and camalexin responded positively to glutathione feeding of *pad2-1*, this result further supports the sulfur donor hypothesis.

Labelling experiments with GS*H or Cys* were performed to test whether GSH or cysteine is the sulfur donor for the thioglucoside linkage, coupling the glucose moiety to the glucosinolate (GS) core structure. When feeding GS*H, we could readily detect incorporation of radioactivity into GS. Moreover, inhibiting the conversion of Cys* to GSH with BSO resulted in lower incorporation. However, the results of these experiments were inconclusive because of the tight connection(s) between cysteine and GSH pools that prevented the exclusive labelling of either pool. It is unclear whether the radiolabel in the cysteine pool directly originated from GS*H and/or from degradation of GS*H-derived products. These experiments were thus unable to produce a definite answer to the sulfur donor hypothesis, and further work will be necessary to determine whether or not GSH is the sulfur donor in GS biosynthesis *in vivo.* The alternative hypothesis is that the activity of GS biosynthetic enzymes is regulated by a GSH-dependent modification.

Measurements of GS levels in Arabidopsis after herbivory have generated contrasting results. It was found that *Spodoptera exigua* feeding causes the accumulation of mainly aliphatic and to some extent indole GS (Mewis *et al.*, 2005, 2006). On the contrary, indole GS but not aliphatic GS content increased 24–72 h after *S. littoralis* feeding (Skirycz *et al.*, 2006). Infestation by the green peach aphid *M. persicae* caused an overall decrease in GS content, whereas the indole GS indolyl-3-methyl-GS was induced (Kim and Jander, 2007). Our results show that *S. littoralis* feeding caused the accumulation of the most abundant indole and aliphatic GS. This indicates some variability in the nature and intensity of GS accumulation depending on the insect species and the experimental conditions. More studies on GS content are required to determine the specificity of the plant response to insects of different feeding guilds.

The defensive role of GS was inferred from insect feeding studies that were difficult to interpret because of the use of artificial diets complemented with compounds of unknown stability. Studies with recombinant inbred lines or signalling mutants showed a negative correlation between GS content and the extent of herbivory (Kliebenstein *et al.*, 2002; Levy *et al.*, 2005; Mewis *et al.*, 2005), but the effect might have been confounded by additional modifications in these genetic backgrounds. The identification of Arabidopsis genes that control GS metabolism has recently allowed more targeted approaches to directly address the role of GS in defence. Double mutant plants *tgg1 tgg2* that have undetectable myrosinase activity were shown to be more attractive to two generalist Lepidoptera, *Trichoplusia ni* and *Manduca sexta*, but were either consumed equally or less by the specialists *Plutella xylostella* or *Pieris rapae*, respectively (Barth and Jander, 2006). In this study, mutants plants with defects in aliphatic GS (*gsm1-1*) or indole GS (*cyp79B2 cyp79B3*) biosynthesis or a defect in the insect-induced accumulation of both types of GS (*coi1-1* and *pad2-1*) all allowed better growth of *S. littoralis* larvae. This provides additional genetic evidence that both indole and aliphatic GS play a crucial role in defence against a generalist herbivore.

GS or their breakdown products have also been investigated in the context of resistance to diverse fungal or bacterial pathogens. It was observed that the fungus *Fusarium oxysporum* was more aggressive on *gsm1-1* than on wild-type plants (Tierens *et al.*, 2001). Production of novel aliphatic or aromatic GS in transgenic Arabidopsis plants conferred increased resistance to the bacterial soft-rot pathogen *Erwinia carotovora* and to *P. syringae* (Brader *et al.*, 2006). The disease susceptibility of *pad2-1* to several fungal or bacterial pathogens (Parisy *et al.*, 2007) might thus be at least partially explained by the insufficient accumulation of camalexin and/or GS.

In summary, we have shown that mutations in various steps of the GS–myrosinase defence system that alter either the expression of GS biosynthesis genes, the synthesis or the breakdown of GS, all result in an enhanced susceptibility to a generalist herbivore (Figure 9). We have identified a new role for GSH in insect resistance and propose that GSH acts

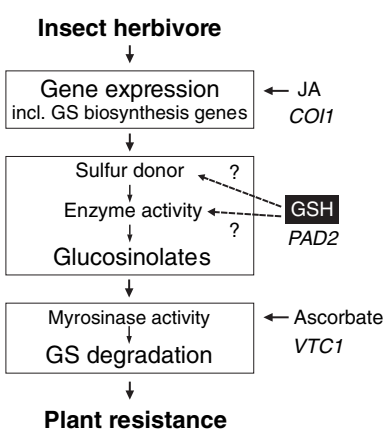

Figure 9. Enhanced susceptibility to generalist herbivores results from defects at various levels of GS metabolism.
This model shows genes that, when mutated in Arabidopsis, result in increased larval performance of *S. littoralis*. PAD2 is a γ-glutamylcysteine synthetase involved in GSH synthesis. Our data suggest that GSH plays a specific role in GS biosynthesis either as a sulfur donor or as a co-factor modulating enzymatic activity. We also show that COI1 controls the expression of GS biosynthesis genes, whereas VTC1 controls myrosinase activity through the synthesis of ascorbate. Plant resistance to insects is also dependent on the expression of other defence genes not shown in this figure that depends in part on a functional COI1 pathway.

either as a sulfur donor or as a specific co-factor in GS biosynthesis. Further research might unravel the precise involvement of GSH in the biosynthesis of secondary metabolites beyond its well-established role as a regulator of redox homeostasis.

Experimental procedures

Plant and insect growth conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Col-0 and mutants were grown as described previously (Reymond *et al.*, 2000). Mutant seeds (*gsm1-1*, *vtc1-1*) were obtained from the Nottingham Arabidopsis Stock Centre. Seeds of *coi1-1* (non-glabrous), *pad2-1* and *pad3-1* were a gift from Jane Glazebrook (University of Minnesota, St Paul, MN, USA), and *cyp79B2 cyp79B3* was obtained from Dr Yunde Zhao (University of California at San Diego, San Diego, CA, USA). Seeds of the male-fertile *coi1-35* were obtained from Dr Paul Staswick (University of Nebraska, Lincoln, NE, USA). Homozygous seedlings of the male sterile *coi1-1* mutants were selected on plates containing 50 μM JA as described previously (Xie *et al.*, 1998), and were transferred to soil after 10 days of growth. Eggs of *Spodoptera littoralis* (Egyptian cotton worm) were obtained from Syngenta, (http://www.syngenta.com), and were stored at 10°C until further use. Two to three days before a bioassay, eggs were placed in a beaker covered with plastic film in a growth chamber (20°C, 65% relative humidity, 100 μmol m^{-2} sec^{-1}, 10/14 h photoperiod) to allow hatching. *Pieris brassicae* (large white butterfly) were reared in a greenhouse on cabbage plants (*Brassica oleracea* cv.). Freshly hatched larvae were used for the bioassays.

Insect bioassays

Insect performance tests with *S. littoralis* and *P. brassicae* were performed as recently described (Bodenhausen and Reymond, 2007). Plants for the GS analyses were grown in a growth chamber (12 h photoperiod, 22°C/18°C, 120 μmol m^{-2} sec^{-1}). For the experiments with the mutants *pad2-1*, *vtc1-1* and *coi1-35*, plants were 4 weeks old and 16 plants per genotype were infested with freshly hatched *S. littoralis*. Plants for the complementation experiment were 5 weeks old and 10 plants per genotype were used. For both experiments, two larvae were placed on five leaves per plant. The various genotypes were randomized in trays without physical contact. Movement of larvae between genotypes was prevented by a water layer at the bottom of the tray. The larvae fed for 48 h and damaged leaves were collected and stored in liquid nitrogen.

Glucosinolate and thiols analyses

For GS extraction, four samples per genotype and treatment were harvested, weighed (200–500 mg), and flash-frozen in liquid nitrogen. Each sample contained one leaf from each of eight different plants and was ground with a glass rod. To each tube, 50 μl of internal standard sinalbin (4-hydroxybenzyl-glucosinolate, 4.152 μmol/ml stock solution, J.C. Sörensen, University of Copenhagen, Denmark; http://www.glucosinolates.com) and 2 ml of 70% v/v methanol were added. After homogenization for 30 sec at full speed (Polytron Kinematica, http:www.kinematica.ch), GS were incubated for 15 min at 80°C. Extracts were centrifuged at 3500 ***g*** for 10 min, and 1 ml of supernatant was loaded on activated DEAE–Sephadex columns. To prepare DEAE–Sephadex columns (Sigma, http://sigmaaldrich.com), 500 μl of A-25 DEAE–Sephadex in 2 M acetic acid was added to 2 ml Eppendorf tubes and was activated with 1 ml of 6 M imidazole formate in 30% formic acid followed by two washes with 1 ml acetate buffer (100 mM, pH 4). After loading, the columns were rinsed twice with 1 ml of acetate buffer (100 mM, pH 4), and 50 μl of partially purified sulfatase (Sigma, http://www.sigmaaldrich.com/; type H-1) (Graser *et al.*, 2001) was added. The GS were desulfated overnight at room temperature on a shaker, and then eluted in 500 μl double distilled H_2O.

Desulfated extracts were separated by HPLC (Bio-Tek, http://www.biotek.com) on a reversed-phase (C18) column (Lichrospher 100, 250 × 4.6 mm, VWR Inc., http:www.merck.de) using ddH_2O as solvent A and acetonitrile (HPLC grade in ddH2O) as solvent B at a flow rate of 1.25 ml/min. Each run consisted of 1% v/v solvent B (1 min), 1–99% v/v solvent B (20 min), 3 min hold at 99% v/v solvent B, 99–1% v/v solvent B (5 min), and a 10 min final hold at 1% v/v solvent B. The eluent was monitored at 230 nm, and GS peaks were identified by comparing retention times relative to standard samples of 4MSB and I3M. Quantification was performed by integrating the peak area for each GS relative to the peak area for the internal standard. Molar concentrations of individual GS were calculated using published response factors (Brown *et al.*, 2003) to correct for absorbance differences between the internal standard and each compound.

GSH and cysteine were extracted and quantified as described previously (Parisy *et al.*, 2007).

Physiological complementation

Rosette leaves (one leaf from eight plants each) were cut from 4–5-week-old plants and placed in Eppendorf tubes with the petioles immersed in H_2O, 1 mM GSH or 1 mM DTT. Two freshly hatched *S. littoralis* larvae were placed on each leaf to induce the biosynthesis of glucosinolates. After 48 h, the larvae were removed, and the leaves were pooled and analysed for glucosinolates (see above).

Labelling experiments

For [^{35}S]GSH and [^{35}S]cysteine labelling experiments, 6-week-old Col-0 plants were challenged with *S. littoralis* for 24 and 48 h, respectively, to stimulate GS synthesis. Leaves were then cut and placed in 24-well plates in a droplet of 30–40 μl containing 0.5 nmol of GS*H (1 mCi/μmol = 1.8×10^6 cpm) or 1 nmol of Cys* (1 mCi/μmol = 13.6×10^6 cpm) (Perkin Elmer, http:www.perkinelmer.ch). After most leaves had absorbed the radioactivity, 500 μl of H_2O were added, and the leaves were left for 24–28 h before measuring the incorporation. For the Cys* feeding experiment, cut leaves were pre-incubated for 4.5 h in various treatment solutions (water control, 1 mM BSO, 3 mM GSH or 1 mM BSO + 3 mM GSH) before the addition of Cys*. At the end of the labelling time, leaves were harvested and analysed by HPLC for thiols and GS (see above). Four leaves were pooled for each sample, and each data point represents three samples that were analysed independently. The compounds of interest were collected and the radioactivity determined (Packard Tri-Carb 2000CA, Perkin Elmer). All the values are corrected for background, extraction and analysis volumes, and are calculated back on a weight basis.

Glucosinolate breakdown assay

Myrosinase activity was assessed by determining the degradation of exogenously added sinigrin (absent in Arabidopsis) in extracts of

the various genotypes. Frozen leaf samples (200–400 mg FW) were pulverized with a cooled glass rod in a 1.5 ml Eppendorf tube and poured into a mortar. The sample was ground using sand without additional liquid and left in the mortar for 3 min at room temperature to degrade endogenous GS. Exogenous sinigrin (4 mg/ml) was supplied as a substrate, adjusting the volume for each sample to one third of its fresh weight (e.g. 100 μl for 300 mg). The extract was further ground to ensure proper mixing, and the reaction was stopped by adding 2 ml MeOH (70%). To all samples, 100 μl sinalbin (1 mg/ml) was added as an internal standard for sinigrin quantification. Glucosinolates were further extracted and analysed as described above.

Microarray experiments and data analysis

For microarray analyses, damaged leaf tissue from 12 challenged plants was harvested at the end of the insect feeding treatment and immediately stored in liquid nitrogen. Leaves from 12 unchallenged control plants were collected at the same time. Total RNA was extracted and labelled according to a previously published procedure (Bodenhausen and Reymond, 2007). A dedicated microarray was constructed containing 292 cDNAs, representing 222 unique genes (Table S1). This microarray included a majority of insect-regulated genes, complemented with pathogen- and abiotic stress-regulated genes and housekeeping control genes (Bodenhausen and Reymond, 2007). All experiments were replicated independently. Hybridization, scanning and data analysis were performed as described previously (Reymond *et al.*, 2000). Up- and down-regulated genes were selected based on a threshold of a twofold change in gene expression and a *P*-value < 0.05. To identify genes differentially expressed by herbivory between genotypes, we performed a Student's *t*-test between $\log_2$-transformed expression ratios from Col-0 experiments and $\log_2$-transformed expression ratios from mutant experiments. To address the issue of multiple comparisons, a false-discovery rate (FDR) was calculated using the method described by Storey and Tibshirani (2003). The FDR at $P = 0.05$ was 0.7% (Col-0), 0.8% (*pad2-1*) and 4.0% (*coi1-1*).

Normalized raw data and mean expression ratios for all genes and all experiments are presented in Tables S1 and S2.

Quantitative PCR

Leaf samples (eight leaves of eight different plants) were harvested 48 h after onset of *S. littoralis* feeding. Total RNA was extracted using the RNeasy Plant Mini Kit, including an RNase-free DNase I treatment (Qiagen, http://www.qiagen.com/). RNA samples were reverse-transcribed using an Omniscript® reverse transcription kit (Qiagen) with 2 μg total RNA. The resulting cDNA samples were diluted fivefold with water. Quantitative RT-PCR was performed in triplicate using ABsolute™ QPCR SYBR® Green mix (ABgene, http:www.ABgene.com) in a Rotor-Gene™ 2000 apparatus (Corbett Research, http:www.corbettlifescience.com). In a 15 μl reaction volume, 5 μl of the cDNA sample was combined with 7.5 μl of 2× SYBR Green mix, 1.6 μl RNase- and DNase-free water, and 0.45 μl of each primer (both at 10 μM). The cycling included 95°C for 15 min, followed by 45 cycles at 95°C for 15 sec, 60°C for 25 sec and 72°C for 20 sec, and finally 72°C for 5 min. The following gene_specific primer pairs were used: reference gene (At4g26410), ref-F (5′-GAGCTGAAGTGGCTTCCATGAC-3′) and ref-R (5′-GGTCCGACATACCCATGATCC-3′); anthranilate synthase alpha 1 (At5g05730), ASA1-F (5′-AACGATGTTGGAAAGGTTACG-3′) and ASA1-R (5′-CGTCCCAGCAAGTCAAACC-3′); cytochrome P450 83B1 (At4g31500), Cyp83B1-F (5′-TTCATGAACGAGCACAAAGG-3′) and Cyp83B1-R (5′-CATTGCAATCCCAAGATGC-3′); sulfotransferase 5a (At1g74100), ST5a-F (5′-ATGGCTGCTCGTATTGATGG-3′) and ST5a-R (5′-CCGCACCAAATAACAGAAGG-3′); putative myrosinase binding protein (At3g16420), MBP-F (5′-TGCTCAGGTTCAAGACTAATAAGC-3′) and MBP-R (5′-TTTTGTGGAGCAGAACATCG-3′). Relative mRNA abundance was calculated using the comparative $\Delta\Delta C_T$ method using the C_T and E values from analysis using Rotor-Gene 4.4 software. The values were normalized to those for the reference gene, and expressed relative to the unchallenged wild-type control sample.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Figure S1. Expression of glucosinolate biosynthetic genes during *Spodoptera littoralis* feeding.

Table S1. Mean expression ratios after *Spodoptera littoralis* challenge.

Table S2. Normalized raw data for all microarray experiments.

Table S3. Feeding experiment with [^{35}S]GSH.

Table S4. Feeding experiment with [^{35}S]cysteine.

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