Major Signaling Pathways Modulate Arabidopsis Glucosinolate Accumulation and Response to Both Phloem-Feeding and Chewing Insects[1]

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Plant responses to enemies are coordinated by several interacting signaling systems. Molecular and genetic studies with mutants and exogenous signal application suggest that jasmonate (JA)-, salicylate (SA)-, and ethylene (ET)-mediated pathways modulate expression of portions of the defense phenotype in Arabidopsis (*Arabidopsis thaliana*), but have not yet linked these observations directly with plant responses to insect attack. We compared the glucosinolate (GS) profiles of rosette leaves of 4-week-old mutant and transgenic Arabidopsis (Columbia) plants compromised in these three major signaling pathways, and characterized responses by those plants to feeding by two phloem-feeding aphids (generalist *Myzus persicae* and specialist *Brevicoryne brassicae*) and one generalist caterpillar species (*Spodoptera exigua* Hubner). Blocked JA signaling in *coronatine-insensitive* (*coi1*) and enhanced expression of SA-signaled disease resistance in *hypersensitive response-like* (*hrl1*) mutants reduced constitutive GS concentrations, while blocking SA signaling at the mediator protein *npr1 mutant* (NPR) increased them. There was no significant impact on constitutive GS contents of blocking ET signaling (at *ET resistant* [*etr1*]) or reducing SA concentrations (*nahG* transgene). We found increased GS accumulation in response to insect feeding, which required functional NPR1 and ETR1 but not COI1 or SA. Insect feeding caused increases primarily in short-chain aliphatic methylsulfinyl GS. By contrast, responses to exogenous JA, a frequent experimental surrogate for insect attack, were characterized by an increase in indolyl GS. Insect performance, measured as population increase or weight increase, was negatively related to GS levels, but we found evidence that other, ET-regulated factors may also be influential. Plant resistance to (consumption by) *S. exigua* was not related to insect growth because some plant chemistries inhibited growth while others inhibited feeding. These major signaling pathways modulate Arabidopsis GS accumulation and response to both phloem-feeding and chewing insects, often antagonistically; NPR appears to be central to these interactions. Our results indicate that exogenous signal application and plant consumption measures may not provide useful measures of plant responses to actual insect feeding.

Plants have developed diverse defense mechanisms for dealing with enemies. Like all plants in the Brassicaceae, Arabidopsis (*Arabidopsis thaliana*) produces secondary metabolites, including glucosinolates (GS), phenolics, and terpenoids (Halkier, 1999; Madhuri and Reddy, 1999; Harrewijn et al., 2001; Mikkelsen et al., 2003), which may have defensive functions. GS are found mainly in the order Brassicales, which includes the Brassicaceae (Giamoustaris and Mithen, 1995; Tierens et al., 2001), where they function in defense against herbivores and pathogens. GS are sulfonated thioglycosides comprising a common glycone moiety with a variable aglycone side chain. Three major classes of GS are distinguished: aliphatic GS derived principally from Met; indolyl GS derived from Trp; and aromatic GS, mostly derived from Phe. GS and hydrolyzing myrosinases (β-thioglucoside glucohydrolase) are compartmentalized and come in contact only upon tissue damage (Koroleva et al., 2000), releasing defensive hydrolysis products including isothiocyantes, nitriles, and epithionitriles (Francis et al., 2001; Kliebenstein et al., 2001). GS substrates are themselves unpalatable to a number of insect herbivores, and the types of hydrolysis products formed are determined by the GS substrate, activity of the epithiospecifer protein, and the reaction conditions (Lambrix et al., 2001). Levels of GS substrates increase and their composition may be altered in response to herbivory and pathogen attack in several Brassicaceae species (Doughty et al., 1991; Bodnaryk, 1992; Mithen, 1992; Hopkins et al., 1998a; Bartlet et al., 1999; Agrawal and Kurashige, 2003; Cipollini et al., 2003; Pontoppidan et al., 2003).

There is ample evidence that GS structures and levels influence host plant suitability for generalist and specialist herbivores (Agrawal and Kurashige, 2003). GS are often repellant to generalist herbivores, and they have become important cues for host plant

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finding and acceptance by GS-adapted specialist insects (Pivnick et al., 1994; Renwick and Lopez, 1999). Increasing the GS levels in *Brassica napus* and *Sinapis alba* reduced the extent of grazing by generalist herbivores but resulted in greater damage by the GS specialist beetle *Psylliodes chrysocephala* and butterfly *Pieris rapae* (Giamoustaris and Mithen, 1995). Differences in susceptibility to herbivores among *S. alba*, *B. napus*, and *Brassica campestris* have been attributed to their GS contents, particularly 4-hydroxybenzyl GS (Bodnaryk, 1991, Hopkins et al., 1998a). A decrease in the side chain length of aliphatic GS and the extent of hydroxylation increased the feeding by *P. chrysocephala* (Giamoustaris and Mithen, 1995).

While the composition, biosynthesis, and genetics of GS have received intense scrutiny in Arabidopsis, there have been few direct studies of their function against insects. Lambrix et al. (2001) found that the generalist herbivore *Trichoplusia ni* (Hübner) fed more readily on nitrile-producing than on isothiocyanate-producing Arabidopsis ecotypesa, but did not associate actual measures of GS in eaten plants with resistance or insect performance. Associations between quantitative trait loci containing genes involved in GS metabolism and susceptibility of some Arabidopsis genotypes to the generalist herbivores *Spodoptera exigua* (Hübner) and *T. ni* (but not the specialist diamondback moth, *Plutella xylostella*; Stotz et al., 2002; Kroymann et al., 2003) indirectly suggest a role for GS in resistance. As far as we know, there are no published studies directly linking herbivore-induced changes in GS with resistance to insects in Arabidopsis.

Plant responses to pests are coordinated by several signaling systems, of which three have received the most attention. One of these, the oxylipin-signaling pathway, includes the hormone jasmonic acid (JA) and related compounds, and has been shown to influence the production of various metabolic defenses, including GS (Titarenko et al., 1997; Kessler and Baldwin, 2002; Mikkelsen et al., 2003). Responses to some pathogens and insects involve a second pathway requiring an interaction between JA and ethylene (ET; Penninckx et al., 1998). Plant response to many microbes involves accumulation of a third signal, the phenylpropanoid salicylic acid (SA), and activation of SA-responsive genes as part of systemic acquired resistance (Glazebrook et al., 2003). There is growing consensus that the three signaling molecules, JA, SA, and ET, interact in complex ways to fine-tune plant defense reactions to the specific stressor (O'Donnell et al., 1996; Reymond and Farmer, 1998; Stotz et al., 2000; Glazebrook et al., 2003). Positive and negative cross talk among these pathways is well established (Pieterse et al., 2001; Cui et al., 2002; Kunkel and Brooks, 2002).

GS accumulation is responsive to exogenous JA and SA (Kiddle et al., 1994; Doughty et al., 1995; Bartlet et al., 1999; Mikkelsen et al., 2003). Suitability for insects also can be altered when JA or SA are applied to Arabidopsis plants or when the insects consume foliage from signal pathway mutants (Stotz et al., 2000, 2002). But it is not clear to what extent plant responses to exogenous signals represent responses to insects. Various insect species may interact with signaling pathways differentially and elicit different responses from their host plants, especially if the insects differ in adaptation to the host plant or feeding style (Stotz et al. 2000, 2002; Moran and Thompson, 2001; Cui et al., 2002). For example, phloem-feeding insects such as aphids, which do little wounding while feeding, may not elicit plant responses typical of chewing insects or wounding (Moran and Thompson, 2001). Preliminary studies suggest that Arabidopsis responses to aphids involve multiple signaling pathways (Moran and Thompson, 2001). Some insects may exploit antagonistic cross talk between signaling networks to alter or suppress plant defense responses (Felton and Korth, 2000; Musser et al., 2002).

Studies of Arabidopsis responses to insects have so far focused on differential gene expression and signaling pathways, usually via use of signaling mutants and application of signaling molecules (e.g. Stotz et al., 2000; Moran and Thompson, 2001; Mikkelsen et al., 2003) but have ignored the chemical defense phenotype, specifically GS composition, arising from actual insect attack. Moreover, the specificity of Arabidopsis' chemical defense response to chewing and phloem-feeding insects has not been compared explicitly in a single study. In this study, we characterized Arabidopsis responses to attack by two phloem-feeding aphids (*Myzus persicae* Sulzer and *Brevicoryne brassicae*) and the chewing insect *S. exigua* using mutant and transgenic plants with modifications in the JA-, SA-, and ET-signaling pathways, and evaluated the impact of constitutive and insect-induced changes in GS on plant consumption and insect growth.

RESULTS

Constitutive GS Levels and Changes in Response to Insects

Quantitative GS profiling indicates that 10 major GS types were present in all plant genotypes. The dominant class of GS in Columbia (Col)-0 leaves was aliphatic methylsulfinyl GS (Fig. 1), of which the most abundant compound was 4-methylsulfinylbutyl GS (4MSOB; Fig. 2). We detected only one GS with a methylthio side chain, 4-methylthiobutyl. Glucobrassicin (3-indolylmethyl [3IM]) was the most abundant indolyl GS, followed by 1-methoxy-3-indolylmethyl (1MO3IM) and 4-methoxy-3-indolylmethyl (4MO3IM; Fig. 2).

The chemistry of the undamaged plants was quite similar in all experiments. The constitutive total GS contents differed among genotypes in experiments with both *M. persicae* and *B. brassicae* and in the experiments with *S. exigua* (two-way ANOVAs $P \leq 0.0001$). Total constitutive GS levels were signifi-

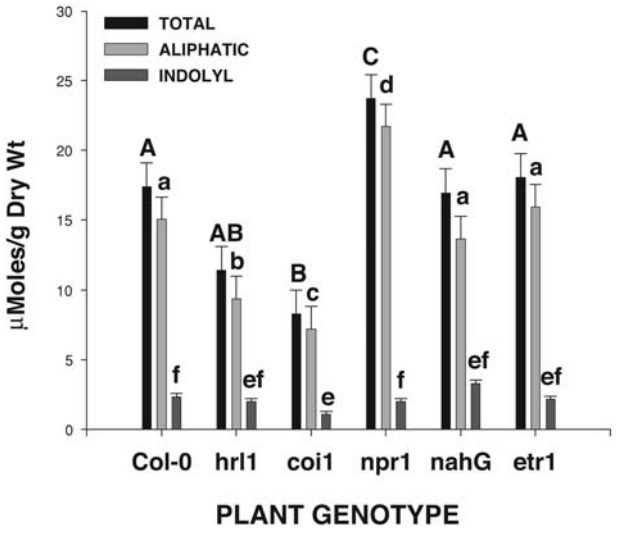

Figure 1. Mean (SE) concentrations of total, aliphatic, and indolyl GS in rosette leaves of 4-week-old Arabidopsis genotypes. Bars within a GS class with the same letter did not differ from each other at $P < 0.05$ (Tukey's HSD tests).

cantly lower in *hypersensitive response-like* (*hrl1*) and in *coronatine-insensitive* (*coi1*) than in the Col-0 controls in both sets of experiments (Fig. 1), while the SA-signaling pathway mutant *npr1* had 40% to 80% greater total constitutive GS levels than found in Col-0 (Fig. 1). In contrast, the constitutive total GS content in the other plant with compromised SA signaling, the transgenic *nahG*, did not differ from Col-0 plants (Fig. 1). The constitutive total GS contents of the ET-insensitive mutant *etr1* were identical to Col-0 in the aphid experiments (Fig. 1) but were 25% higher in the caterpillar experiments (data not shown), evidently due to slight differences in plant age and growth conditions. Differences among the genotypes in total constitutive GS contents were due mainly to differences in aliphatic GS. All of the mutants generally had either the same or slightly but significantly lower indolyl GS levels than did Col-0 (Fig. 1).

Total Col-0 GS levels increased significantly in response to feeding by *M. persicae*, *B. brassicae*, and *S. exigua* (Figs. 3 and 4). One week of feeding by either aphid species produced increases of 16% to 18% in total GS content compared to controls, whereas the caterpillar elicited a 2-fold increase within 1 d. Increases in total GS levels elicited by aphid feeding were due almost entirely to increases in the amount of short-chain aliphatic methylsulfinyl GS such as 3-methylsulfinylpropyl and 4MSOB (Fig. 2). *S. exigua* elicited significant increases in short-chain aliphatic GS and 8-methylsulfinyloctyl (8MSOO).

Feeding by the specialist aphid *B. brassicae* often elicited stronger responses than did the generalist *M. persicae*. Slight but significant increases in indolyl GS fractions were seen only in *npr1* (in response to *B. brassicae*) and *etr1* (in response to both aphids, Fig. 3); *S. exigua* never elicited indolyl GS responses (Fig. 4).

The ability to respond to insect feeding with increases in total GS content required a functional NPR1 and ETR1; responsiveness to all three insect species was abolished in *npr1* and *etr1* (Figs. 3 and 4). *nahG* plants were very responsive to insect feeding, as was *coi1*, despite having reduced constitutive total GS levels (Figs. 3 and 4).

GS Changes in Response to JA Treatment

Because published studies have found other Brassicaceae to respond to insect feeding primarily with increases in indolyl GS (Bodnaryk, 1992; Doughty et al., 1995; Hopkins et al., 1998b; Bartlet et al., 1999) and many authors have used exogenous JA to simulate insect attack or elicit defense responses (e.g. Brader et al., 2001, Winz and Baldwin, 2001; Stotz et al., 2002; Mikkelsen et al., 2003), we examined the impact of exogenous JA on GS content of sibs of the wild-type plants used in our experiments. Col-0 plants

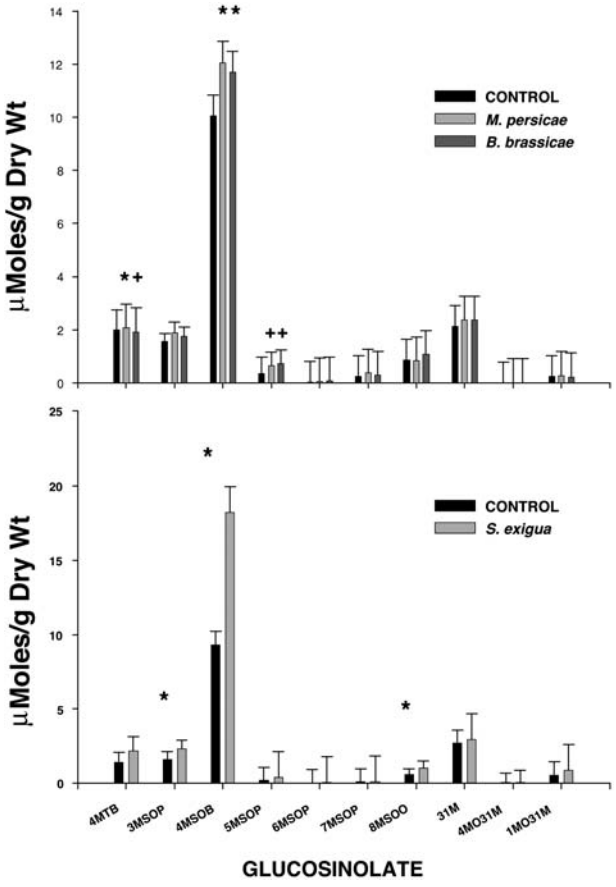

Figure 2. Mean (SE) concentrations of individual GS species in undamaged and aphid-attacked (top) or caterpillar-attacked (bottom) rosette leaves of 4-week-old Arabidopsis Col-0 plants. Bars with an asterisk (*) differed statistically from undamaged controls at $P < 0.05$; those with a plus sign (+) differed at $P < 0.07$ (Tukey's HSD test). Aliphatic GS are 4-methylthiobutyl (4MTB), 3-methylsulfinylpropyl (3MSOP), 4MSOB, 5-methylsulfinylpentyl (5MSOP), 6-methylsulfinylhexyl (6MSOH)/7-methylsulfinylheptyl (7MSOH), and 8MSOO. Indolyl GS are 3IM, 4MO3IM, and 1MO3IM.

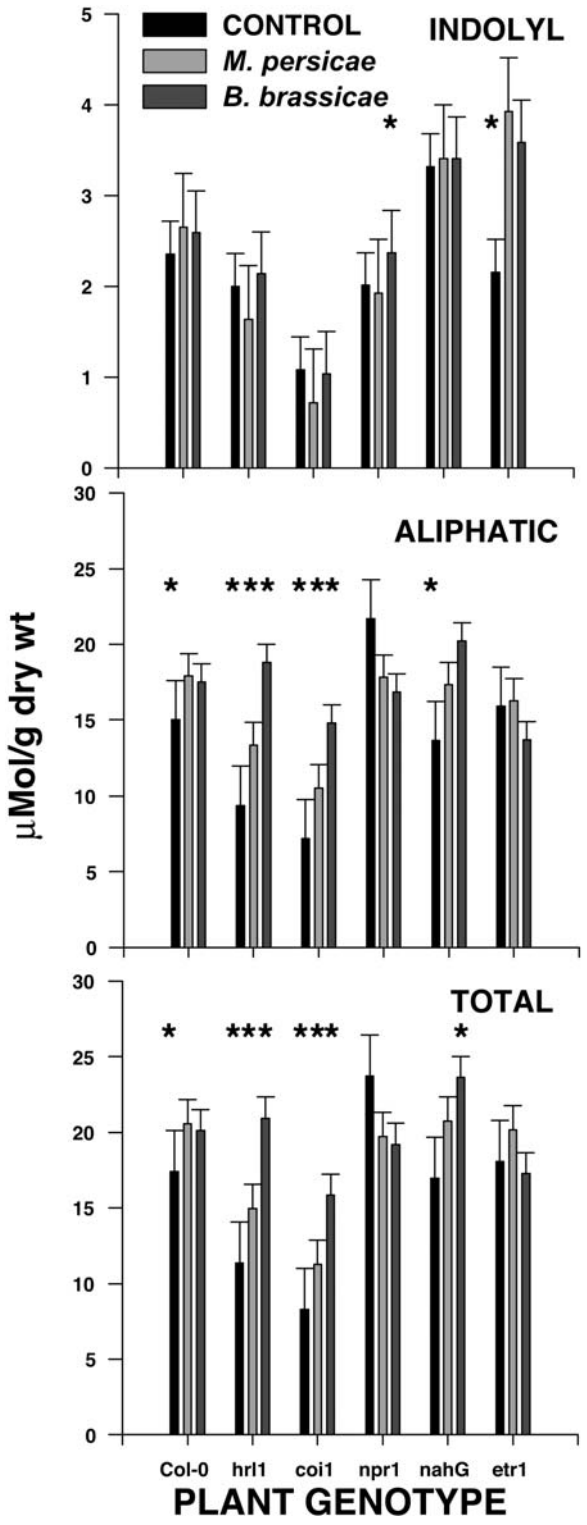

Figure 3. Mean (SE) concentrations of total, aliphatic, and indolyl GS in leaves of undamaged and aphid-attacked leaves of Arabidopsis Col-0 and signaling-constrained plants. Asterisks indicate insect-induced differences from undamaged controls significant at $P < 0.05$ (Tukey's HSD test).

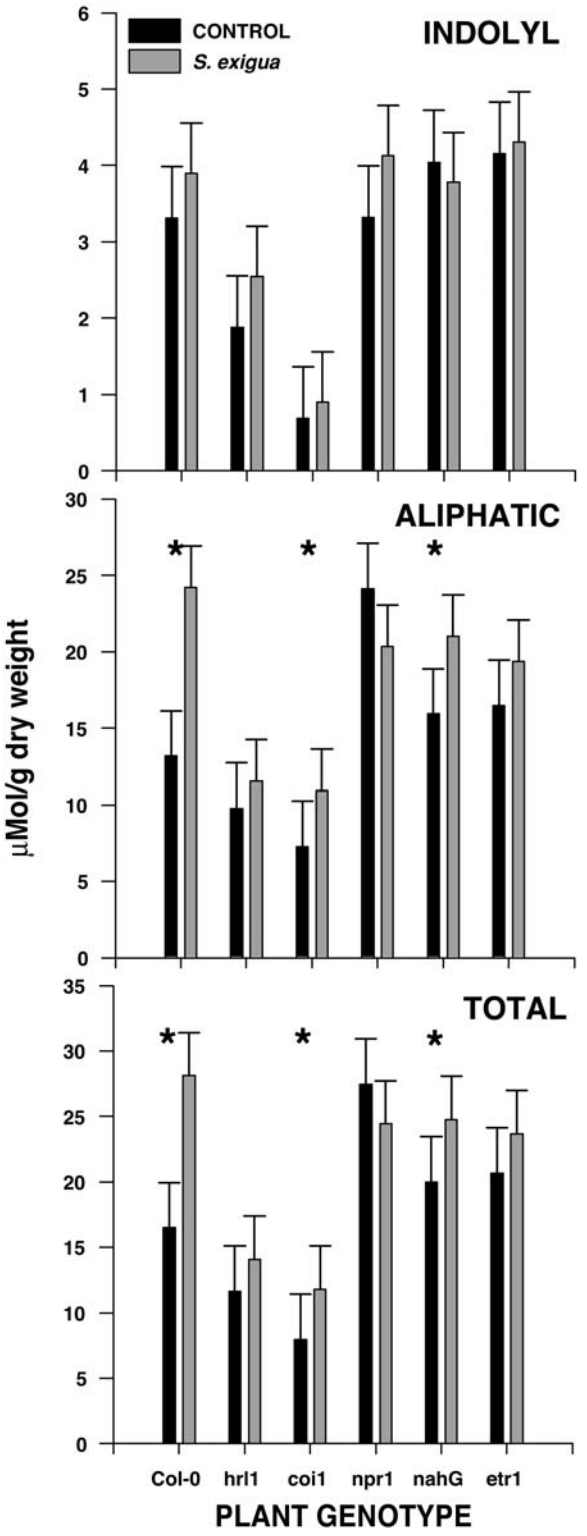

Figure 4. Mean (SE) concentrations of total, aliphatic, and indolyl GS in leaves of undamaged and *S. exigua*-attacked leaves of Arabidopsis Col-0 and signaling-constrained plants. Asterisks indicate insect-induced differences from undamaged controls significant at $P < 0.05$ (Tukey's HSD test).

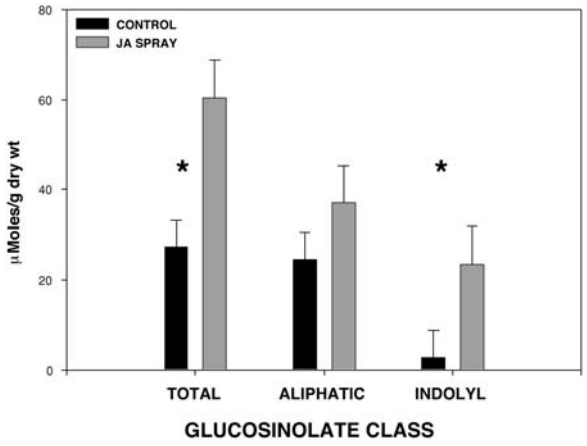

Figure 5. Mean (SE) concentrations of total, aliphatic, and indolyl GS in leaves of Arabidopsis Col-0 plants sprayed with solvent and plants sprayed with 0.25 mM JA. Comparisons with asterisk differed at $P < 0.05$ (Tukey's HSD test).

exhibited a 2-fold increase in total GS content following treatment with 2.5 mM JA compared to solvent-treated control plants (Fig. 5). Unlike the highly significant increase in short-chain aliphatic GS in response to insects in our experiments, exogenous JA caused large increases in the indolyl GS fraction. The greatest increase was observed for 1MO3IM, which increased 100-fold compared with untreated plants; 3IM and 4MO3IM increased 4- to 5-fold. The total aliphatic GS content did not increase significantly after JA treatment, although levels of 5-methylsulfinylpentyl and 8MSOO increased significantly (data not shown).

Plant Resistance and Insect Performance

We assessed plant resistance to *S. exigua* as leaf area removed over a 24-h period and *S. exigua* performance as mass accumulated during the same interval (by the same insects). Resistance and impact on aphids together were assessed as aphid population growth on the various genotypes. We then examined relationships between insect performance and constitutive and elicited GS levels.

B. brassicae and *M. persicae* populations increased about 4-fold in 1 week on Col-0 (Fig. 6). Reproduction of both aphid species differed significantly among the different Col genotypes, and the genotypes influenced performance of the two species differentially. Populations of *B. brassicae* (the specialist) increased significantly more rapidly on *coi1* but significantly more slowly on *npr1* and *nahG* (Fig. 6). *B. brassicae* performance on *hrl1* and *etr1* did not differ from that on Col-0.

Populations of *M. persicae* (the generalist) grew significantly more rapidly on *hrl1* and growth was marginally (ANOVA $0.1 > P > 0.05$) greater on *coi1*. *M. persicae* populations grew significantly more slowly on *npr1* and *nahG*. Growth on *etr1* did not differ from that on Col-0 (Fig. 6).

There also was significant variation among plant genotypes in their impact on *S. exigua* weight gain (Fig. 7). *S. exigua* larvae gained significantly more weight on *coi1* and *hrl1* than on Col-0 plants. Larvae on *hrl1* and *coi1* had 50% to 100% greater weight gain than larvae on Col-0, despite consuming less than half as much leaf material. Larval growth was worst on the two genotypes with blocked SA signaling, *npr1* and *nahG*, followed closely by growth on *etr1*, in which ET

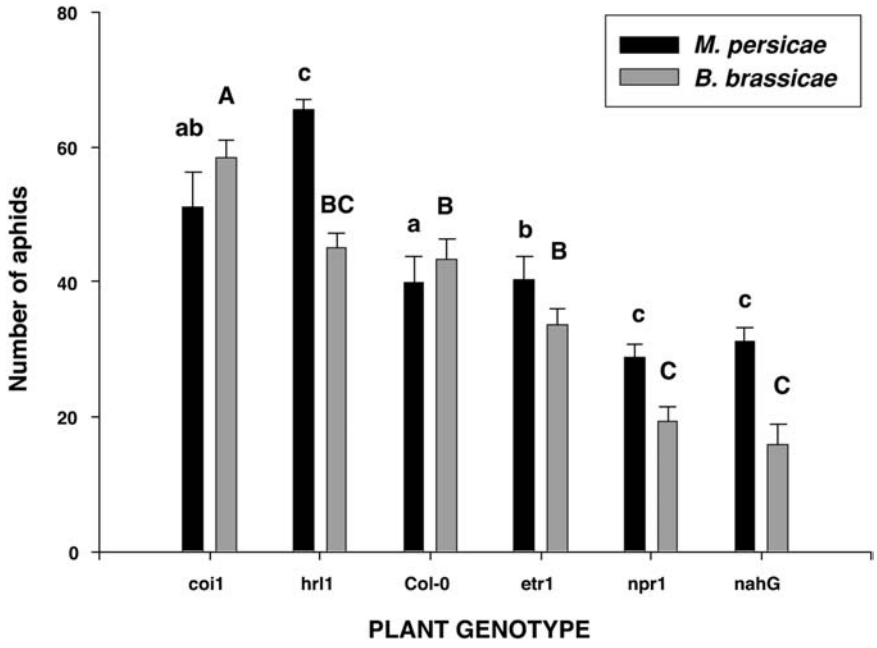

Figure 6. Final population sizes of the generalist aphid *M. persicae* and the specialist aphid *B. brassicae* after 10 d on each of the Arabidopsis genotypes. Bars with different letters within an aphid species identify population sizes that differed at $P < 0.05$ (Tukey's HSD test).

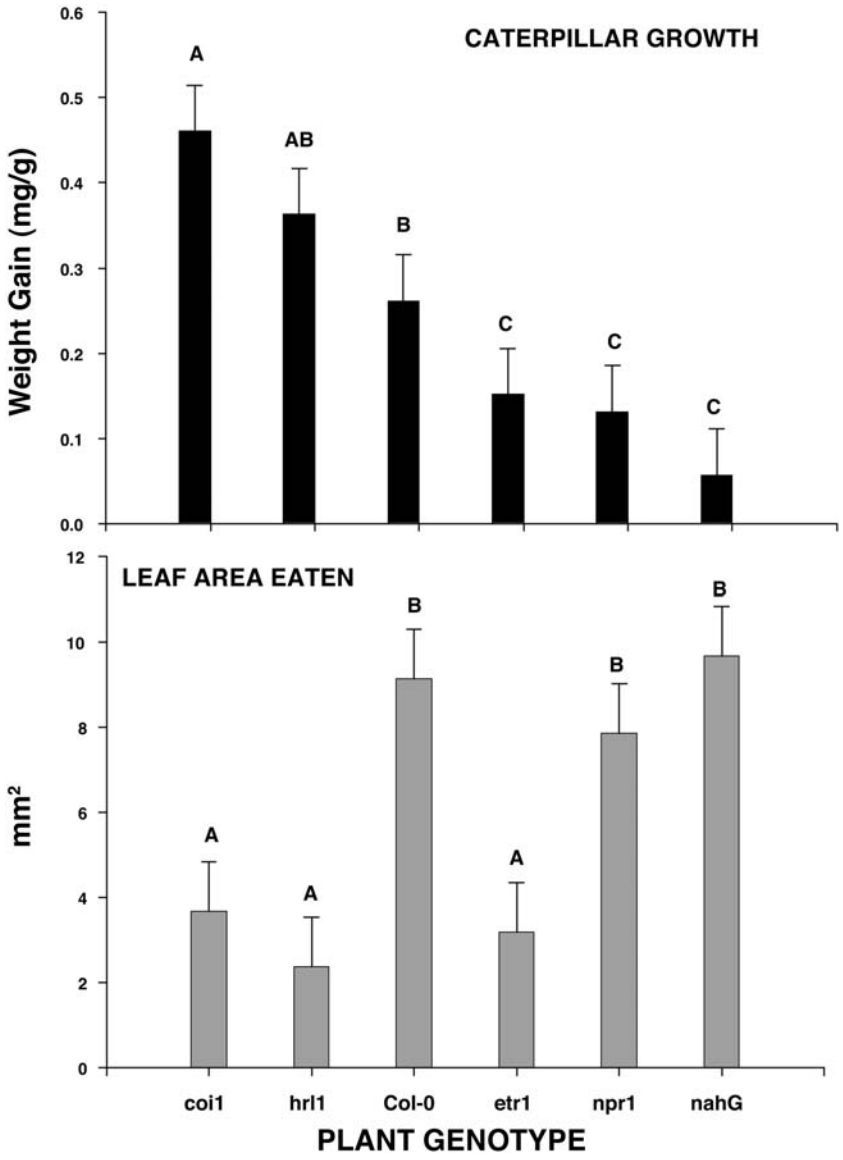

Figure 7. Mean (SE) consumption (bottom) and weight gain (top) by *S. exigua* caterpillars over 48 h on each of the Arabidopsis genotypes. Bars within a measure (consumption, growth) having different letters differed at $P < 0.05$ (Tukey's HSD test).

signaling is blocked (Fig. 7). Weight gain on *nahG* plants was only 20% of that on Col-0 and only 11% of that on *coi1*. Weight increase on *etr1* was about 60% of that on Col-0.

Plant resistance to *S. exigua*, evaluated as fraction of leaf area consumed, also differed significantly among the genotypes (Fig. 7). *hrl1*, *coi1*, and *etr1* were significantly more resistant (less was consumed) than Col-0. Consumption of *npr1* and *nahG* could not be distinguished statistically from consumption of Col-0. Consumption of individual genotypes and larval weight increase on those genotypes were often inversely related (Fig. 7). For example, the best insect growth was achieved on *coi1*, which was one of the least-consumed plants, while the reverse was true for *nahG* (Fig. 7).

Relationship between Insect Performance and GS Contents

We used simple correlation to explore the likelihood that constitutive or induced GS levels we observed in experimental plants might be a basis of resistance and insect performance (Fig. 8). The final number of aphids and the plants' constitutive GS content were negatively related ($R_{\text{constitutive}} = -0.733$ for *B. brassicae* and $R_{\text{constitutive}} = -0.711$ for *M. persicae*; this relationship was stronger than that with induced GS levels, especially for *B. brassicae* ($R_{\text{induced}} = -0.316$ for *B. brassicae* and $R_{\text{induced}} = -0.625$ for *M. persicae*). Aphid performance was somewhat more strongly correlated with aliphatic GS than with indolyl GS ($R_{\text{aliphatic}} = -0.571$ versus $R_{\text{indolyl}} = -0.486$ for *B.*

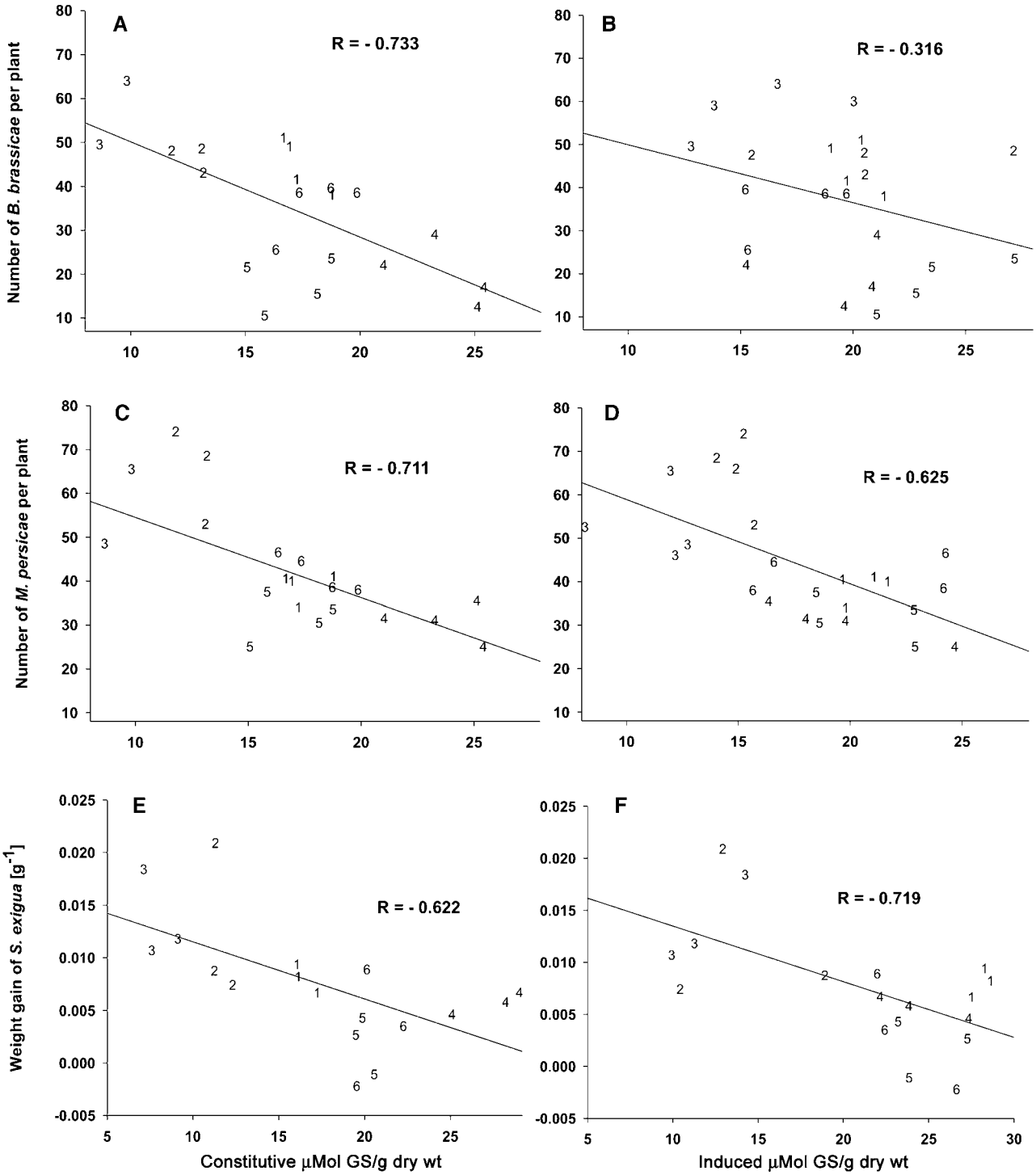

Figure 8. Pearson product-moment correlations between aphid population growth or *S. exigua* weight gain and the total constitutive (A, C, E) or induced (B, D, F) GS concentrations of the plants on which they fed. Numbers refer to the plant genotypes: 1, Col-0; 2, *hrl1*; 3, *coi1*; 4, *npr1*; 5, *nahG*; and 6, *etr1*.

brassicae and $R_{aliphatic} = -0.514$ versus $R_{indolyl} = -0.359$ for *M. persicae*).

Weight gain by *S. exigua* was negatively related to both constitutive and induced total GS levels ($R_{constitutive} = -0.559$ and $R_{induced} = -0.748$). Consumption was not related to constitutive GS levels but was positively related to total induced aliphatic GS concentrations ($R = 0.690$) due to the induction caused by feeding. The performance of *S. exigua* was related about equally to aliphatic and indolyl GS

concentrations ($R_{induced} = -0.691$ for aliphatic GS and $R_{induced} = -0.614$ for indolyl GS).

DISCUSSION

In Arabidopsis, the signaling molecules SA, JA, and ET interact in complex ways to influence plant resistance to herbivores and pathogens (Pieterse et al., 2001; Glazebrook et al., 2003). Plant genotypes affected in their response by any of these signal molecules may become more suitable or resistant to insect herbivory, depending on the herbivore and experimental details (Cui et al., 2002; Stotz et al., 2002). Here, we have shown that modifications of signaling pathways in Arabidopsis ecotype Col lead to changes in constitutive GS levels, GS induction by insect feeding, and suitability for representatives of two major classes of insect herbivores.

Normal Constitutive GS Accumulation Requires Functional COI1 and NPR1

The *coi1* mutant, which has impaired oxylipin signaling, had a lower total GS content than did Col-0, due to reductions in both aliphatic and indolyl GS. This is consistent with a dependence on JA signaling for indolyl and aliphatic GS accumulation as suggested by responses to JA application in this study and in others (Bartlet et al., 1999; Mikkelsen et al., 2003). Presumably, reduction or loss of JA signaling produced the lower GS concentrations we observed in *coi1* plants. Kloek et al. (2001) concluded that SA signaling is normally suppressed by COI1 because SA-mediated events are up-regulated in *coi1* plants. Mikkelsen et al. (2003) elicited greater increases in indolyl GS with exogenous methyl JA than with an SA analog, and application of both signals together suppressed responses. Since the JA and SA pathways are thought to be mutually antagonistic (Gupta et al., 2000; Kunkel and Brooks, 2002; Glazebrook et al., 2003), reduced GS levels in *coi1* may come about via reduced stimulation of GS production by JA, enhanced suppression by SA, or both.

According to this antagonistic cross talk model, blocking SA signaling should increase total GS levels (Mikkelsen et al., 2003). As expected, the *npr1* mutants, in which SA signaling is blocked, produced significantly greater amounts of aliphatic and total GS than found in Col-0 either before or after insect feeding. Indolyl GS in *npr1* plants did not differ significantly from Col-0.

The Arabidopsis *hrl1* mutant is characterized by constitutively expressed SA-regulated (e.g. *PR-1*) and JA-regulated (e.g. *PDF.1.2*) genes, elevated levels of SA and ET, and enhanced resistance to virulent bacterial and oomycete pathogens (Devadas et al., 2002). We found that the *hrl1* mutant has a lower constitutive total GS content than Col-0, but no reduced indolyl GS fraction as found in *coi1*. This is consistent with the negative cross talk model as supported by Mikkelsen et al. (2003), who observed reduced GS levels of Col-0 plants after treatment with an SA analog and JA applied together.

However, blocking accumulation of SA itself while NPR1 is intact had no impact on total GS accumulation. Total, aliphatic, and indolyl GS levels in transgenic *nahG* plants, which have low SA levels, were not statistically distinguishable from those in Col-0. This suggests that antagonism of at least some JA-mediated defense events by the SA pathway does not actually involve SA, despite the fact that some JA-mediated traits can be suppressed with exogenous SA (e.g. Cipollini et al., 2003, Mikkelsen et al., 2003). Impaired SA signaling should allow increases in at least indolyl GS concentrations, since these are stimulated by exogenous JA. However, untreated *nahG* plants exhibited only slightly, statistically insignificant, elevated 3IM and 1MO3IM, which were not enough to alter total GS concentrations. At least some impacts of the SA pathway on GS production arise downstream of SA accumulation, evidently involving NPR1, suggesting that inferences from exogenous SA application have limited value. The *etr1* mutation did not exhibit statistically significant impacts on total GS concentrations under our experimental conditions.

Arabidopsis Responds to Phloem-Feeding and Chewing Insects with Altered GS Levels

We found that 24 h of feeding by the chewing insect *S. exigua* and 1 week of feeding by the phloem-feeding insects *M. persicae* and *B. brassicae* alters GS levels in Col-0, although to different extents. Col-0 responded to feeding by all three insect species with increases in short-chain aliphatic methylsulfinyl GS, and the longer chain 8MSOO increased in response to *S. exigua*; indolyl GS were not affected significantly. These results demonstrate biochemical defense responses to insects in Arabidopsis. This finding is unlike several studies of responses to insects in other members of the Brassicaceae, which have focused on changes in indolyl GS (Bodnaryk, 1992; Hopkins et al., 1998b; Bartlet et al., 1999), possibly because Arabidopsis GS composition is dominated by aliphatic, not indolyl, GS (Kliebenstein et al., 2001).

Our results also contrast markedly with the induction of indolyl GS in response to exogenous JA found in some studies (e.g. Stotz et al., 2000; Mikkelsen et al., 2003) and confirmed in this study. Exogenous JA application usually elicits dramatic increases in concentrations of indolyl GS; impacts on aliphatic GS are inconsistent. Applying 25 mM JA to our Col-0 plants produced significant increases in total indolyl GS concentrations and significant increases in two aliphatic GS but not in total aliphatic GS concentrations. All three insects elicited increases in aliphatic GS concentrations. Only the aphid *B. brassicae* elicited increases in individual, but not total, indolyl GS. These

findings suggest that JA application is not a suitable surrogate for actual insect attack in Arabidopsis (Col).

There may be several reasons why responses to insects differ from responses to exogenous hormone treatments. First, the literature does not present a uniform picture of GS elicitation by exogenous JAs. For example, Mikkelsen et al. (2003) found that methyl JA increases both indolyl and aliphatic GS, while Brader et al. (2001) found induction only of indolyl GS. Second, many papers do not report statistical support for their conclusions. Third, heterogeneous results may arise from varied methods and materials. We sprayed plants with 97% pure 2.5 mM (±)-1a,2b-3-Oxo-2-(cis-2-pentenyl)cyclopentaneacetic acid or JA. Most other investigators (e.g. Brader et al., 2001; Stotz et al., 2002; Mikkelsen et al., 2003) state that they used methyl JA but do not provide racemic composition or source information. It is not clear how responses to methyl JA may differ from responses to JA or to insects (Beale and Ward, 1998). Experimenters also have applied different amounts, often differing by 2-fold or more, over various time courses. Fourth, except for only a few studies, the internal concentration of JA is never measured so the effect of exogenous application on internal concentrations is unknown and likely to be influenced by plant age and metabolic activity. Even if exogenous JA application were relevant to herbivory, differing hormone compositions and applications are unlikely to provide a consistent picture. Finally, our results support the view that plant responses to insects involve a complex interaction among multiple signaling systems; it is not surprising that applying a single signal does not reproduce these effects. Our data clearly indicate that Arabidopsis plants respond differently to insects than to exogenous JA.

Arabidopsis responses to the aphids were significant but quantitatively less intense than to *S. exigua* caterpillars. Aphids do relatively little wounding and have been shown to induce expression of several SA pathway-regulated genes such as *PR-1* and *BGL2*, an effect that could suppress activity in the JA pathway (Moran and Thompson, 2001; Mayer, et al., 2002). Either lack of wounding or cross talk between SA and oxylipin pathways elicited by the aphids might explain reduced plant responses to aphid feeding.

We found that GS responses to all three insects required a functional NPR1 and ETR1 but not SA or COI1. These results support the view that NPR1 is a point of intersection of multiple signaling pathways, especially JA- and SA-mediated antagonism (Pieterse et al., 2001; Cui et al., 2002; Kunkel and Brooks, 2002). Evidently this intersection can be activated by SA-independent as well as SA-dependent means. Our results also indicate that while some responses to insects or pathogens may be mediated by JA or other oxylipins, COI1 is not required for GS responsiveness, since GS increased significantly from low constitutive levels when insects fed on *coi1* plants. Since ETR1 was required for response to insects, it may lie directly in a signaling pathway activated by insect attack, or it may modulate the activities of SA- and JA-mediated pathways. More studies of the relationship between ET signaling and SA/JA antagonisms, especially with double-mutant constructs, are needed.

Insect Performance and Plant Resistance Are Influenced by Signaling Pathways and GS Content

Aphid performance was influenced significantly by plant genotype and insect species. Populations of both aphid species grew relatively poorly on the genotypes with impaired SA signaling (*npr1* and *nahG*), suggesting that SA signaling normally suppresses plant resistance to these aphids, possibly by inhibiting JA-signaled events. Moran and Thompson (2001) obtained similar but less consistent results. In their experiments, *M. persicae* aphids performed better on *npr1* than on wild-type plants in one experiment, but worse in another experiment. This variation may have arisen from much greater aphid densities in their studies, which makes relationships between plant quality and density more difficult to assess, and the fact that their aphids fed on cauline leaves as well as rosettes, while we worked only with rosette (i.e. nonbolting) plants. We find that even subtle differences in plant age, development, and environmental conditions can alter bioassay outcomes (J.C. Schultz, unpublished data). Bioassays must be performed with physiologically and numerically similar organisms to be comparative. Our results are consistent with those of Ellis et al. (2002), who found that *M. persicae* grew less well on the mutant *cev-1*, which has enhanced JA signaling, than on wild-type plants, and grew better on *coi1-16*, with impaired JA signaling, but we did not work with either of those mutants.

Aphid performance in our studies was negatively related to both constitutive and insect-induced GS levels, but constitutive chemistry had the stronger impact on population growth by both aphid species. *npr1* had the highest constitutive GS contents and responded to *B. brassicae* feeding with elevated indolyl GS levels but not aliphatic GS, while *nahG* had constitutive wild-type total GS levels but produced the highest total GS levels after aphid feeding. Both aphid species performed comparatively better on *coi1*, in which JA signaling is blocked and constitutive GS levels are low. Correlations confirmed this picture: Aphid population increase was negatively related to total constitutive and induced GS levels across all plant genotypes. The pattern of aphid performance across genotypes is consistent with a model in which their success depends to some extent on SA pathway suppression of JA-signaled events, which include GS production. However, our results indicate that these interactions are not reproduced correctly by exogenous JA or SA, since these treatments do not produce either the patterns in constitutive GS composition seen in the mutants or the changes in aliphatic (and not indolyl) GS elicited by actual insect feeding.

Some responses and impacts were aphid species specific. For example, *hrl1* plants responded strongly to *B. brassicae* with increased total and aliphatic GS production, much as did Col-0, and *B. brassicae* performance was the same on the two. But *hrl1* responses to *M. persicae* were weaker, and *M. persicae* performed better on those plants than they did on Col-0. *B. brassicae* benefited more than did *M. persicae* from blocked JA signaling and reduced GS levels in *coi1*, even though *B. brassicae* elicited a stronger response. These detailed differences among plant genotypes in response to the two aphid species produced a statistically significant overall difference in the pattern of response to the two insect species. Different responses by the signaling mutants suggest that the similar GS chemistry responses to the two aphid species by Col-0 may have arisen via different signaling networks. Differential performance by the two aphid species also may reflect differing tolerance of plant defenses.

As was true for the aphids, *S. exigua* results were consistent with a model in which insect success depends to some extent on SA pathway suppression of JA-signaled events, including GS production. For example, while *coi1* plants were responsive to *S. exigua* feeding, their leaves began with significantly suppressed GS concentrations, so insect-elicited increases never even reached unwounded Col-0 levels, and *S. exigua* performed significantly better on them than on Col-0 despite consuming significantly less. However, while plant resistance and aphid performance are functionally linked (greater aphid population increase reflects low plant resistance), resistance can be uncoupled from chewing insect performance because insect growth is influenced by consumption, food suitability, or both. The *coi1*, *hrl1*, and *etr1* mutants were most resistant (consumed least), but the best *S. exigua* weight gain occurred on *coi1* and *hrl1*, while *S. exigua* grew little on *etr1* because they refused to eat it. By contrast, *S. exigua* gained very little weight on *npr1* and *nahG* despite consuming substantial amounts. Poor *S. exigua* performance on *npr1* may be explained by very high constitutive GS levels, while performance on *nahG* may be related to the strong increase in GS levels in response to insect attack. Alternatively, insect performance on *nahG* plants could have been reduced by high levels of catechol produced by the bacterial SA hydroxylase; GS extracts of *nahG* were very pink, indicating a high flavonoid content. Catechol and other phenolics are often insecticidal (Felton et al., 1992; Duffey and Stout, 1996). We agree with van Wees and Glazebrook (2003) that *nahG* is not a useful model for assessing the biological/ecological impacts of reduced SA concentrations.

Plant impacts on *S. exigua* performance may also reflect an impact of signal cross talk. We found the greatest insect weight increase on plants with impaired JA signaling and the worst on plants with impaired SA signaling. Cui et al. (2002) reported reduced performance of the generalist caterpillar *T. ni* on genotypes compromised in SA signaling (*npr1*, *nahG*, *pad4*, *eds5*, and *sid2*) and enhanced growth on plants with elevated SA levels (*cpr1* and *cpr6*). Similarly, Stotz et al. (2002) observed reduced performance of *Spodoptera littoralis* (Boisd.) on *npr1* and enhanced performance on *coi1*. The *hrl1* mutant used in our study exhibits increased expression of genes normally up-regulated by exogenous SA, enhanced disease resistance, and elevated SA levels (Devadas et al., 2002); improved *S. exigua* growth on these plants may arise from SA suppression of normal oxylipin signaling, which was generally associated with reduced GS production and enhanced larval growth across the genotypes we studied.

Differences among published studies of insects feeding on Arabidopsis and related species call attention to the need for comprehensive studies linking signaling, plant defense phenotype, and ecological outcomes, particularly similar measures of resistance. Compensatory feeding is well established (Price et al., 1980): Insects confined to low-quality plants commonly compensate for quality by consuming more of plants they might avoid given a choice. Different means of assessing plant resistance thus can provide dramatically different results. Hence, insect growth as a function of consumption calculated from no-choice and choice-based assays cannot be compared. Growth and consumption data must instead come from the same plants and experiments and must be measured in the same way (either choice or no choice) to be biologically, logically, and statistically valid.

Since we measured consumption as leaf area removed (as have all previous studies), it is possible that differences in specific leaf mass could produce differences in total amount (as mass) of plant consumed. We measured specific leaf mass of two plant genotypes differing dramatically in consumption and caterpillar growth and the only two seeming to differ in leaf thickness, *hrl1* and Col-0. *hrl1* leaves had lower specific mass (mean of 3.7 mg cm^{-2}, $n = 9$) than did Col-0 (4.24 mg cm^{-2}, $n = 9$). However, *hrl1* was least consumed and yet supported best insect growth, the opposite of expectations if more leaf area was consumed to compensate for low mass. We are satisfied that leaf area removed is a valuable measure of plant loss or resistance in this study. The need to consume more leaf area to acquire sufficient mass is just another reason why insect growth and plant consumption (resistance) are frequently uncoupled, as we observed in this study.

Many factors influence insect feeding behavior and physiology. Feeding preferences are frequently conditioned by diet immediately prior to feeding on a particular plant, so switching insects from artificial diet to Arabidopsis for experiments without a period of acclimation to the new diet may not produce realistic results (Renwick, 2001). Even closely related insect species often have differing evolutionary and developmental histories and perceive the same plants in different ways. GS chemistry differs dramatically among Arabidopsis rosette leaves of different ages (Petersen et al.,

2002; Brown et al., 2003), so that plants that differ in age may present different defensive traits to herbivorous insects, producing different feeding behaviors.

Plant responses to insects together with insect behavior comprise a complex system. To understand plant-insect interactions, we must characterize the relationships among the defense phenotype, signaling pathways, gene expression, and differential feeding and growth (phenotype) using insects actually feeding on the same plants under identical conditions, with statistical support. Attempts to accomplish this have so far been piecemeal and may provide misleading results. We suggest that studies that focus simultaneously on several layers in the complex interaction between insects and plants are needed to avoid unwarranted generalizations.

The impact of ET signaling on caterpillars may involve mechanisms other than GS production. Our results are in agreement with several studies in which intact ET signaling appeared to enhance food quality or susceptibility of plants for chewing insects. Stotz et al. (2000) demonstrated reduced consumption of the ET-signaling mutant *ein2* by the dietary-generalist caterpillar *S. littoralis* and increased consumption when Col-0 plants were pretreated with ethephon. Winz and Baldwin (2001) showed that ET signaling appears to interfere with plant defense in tobacco (*Nicotiana tabacum*), increasing consumption by the specialist caterpillar *Manduca sexta*. Disrupted ET signaling in *etr1* also significantly reduced consumption and the quality of the plant as food for *S. exigua* in our studies, despite GS levels that were very similar to Col-0 and were not inducible by this insect. Evidently, some other resistance trait influenced by ET signaling is important to *S. exigua*.

While many plant traits, both constitutive and induced, may influence resistance to enemies and enemy performance, our results strongly suggest that both constitutive and inducible variation in GS contents and composition mediated by SA, JA, and ET signaling contributed significantly to differences in insect performance across plant genotypes. While we focused on GS substrates, the biological activity we observed probably also reflects the ratio of GS hydrolysis products the insects experienced. Two myrosinase isoenzymes from *B. napus* were shown to degrade aliphatic GS at higher rates than indolyl GS (James and Rossiter, 1991). Since the Col ecotype supposedly does not express the epithiospecifier protein (Lambrix et al., 2001), the main hydrolysis products arising from the dominant aliphatic GS should be isothiocyanates, which can have a greater negative impact on insects than do the indolyl-derived nitriles (Eckardt, 2001; Lambrix et al., 2001). Our findings also indicate that other plant resistance traits (e.g. phenolics, as suggested by the *nahG* results) and the separate functions of repellancy and antibiosis need to be investigated in Arabidopsis. Our results suggest a central role for *NPR1* in constitutive total GS accumulation in Arabidopsis ecotype Col, as an intersection point among the signaling pathways we studied, a view consistent with the suggestion of Spoel et al. (2003). In addition, the perception of ET at *ETR1* is required for aliphatic (at least) GS induction by insects. Few, if any, of our results would be duplicated by exogenous application of these putative signals.

We have linked genotypic, signaling, and phenotypic (chemical defense) variation with resistance and insect performance in Arabidopsis. This kind of variation is the basis of ecological and evolutionary interactions between plants and insect herbivores. Most variation among genotypes and in responses to insects occurred among the aliphatic GS, suggesting that this GS class warrants increased attention in the context of resistance to insects. While some of our results are consistent with previous work in the Brassicaceae, others suggest that not all generalizations can be extended to Arabidopsis. While study at each level of plant response to insects is important and useful, formulating strong generalizations will require comparative, coordinated, simultaneous study at multiple levels using carefully characterized plants and insects.

MATERIALS AND METHODS

Plant Material and Cultivation

Four Arabidopsis (*Arabidopsis thaliana* ecotype Col) signaling pathway mutants (*hrl1*, *coi1*, *npr1*, and *etr1*), the transgenic *nahG*, and the corresponding Col wild type (Col-0) were used. Seeds of *coi1-1* (glabrous) were obtained from Dr. John Turner (University of East Anglia, Norwich, UK), *etr1* from the Arabidopsis Biological Resource Center, Columbus, Ohio, *npr1* from Dr. Xinnian Dong (Duke University, Durham, NC), and nahG from Paradigm Genetics, Research Triangle, North Carolina. These plants had the following modifications of their signaling pathways: *hrl1*, constitutive SA- and JA-mediated responses, elevated SA concentrations, constitutive resistance to virulent *Pseudomonas syringae* and *Peronospora parasitica* (Devadas et al., 2002); *coi1*, insensitive to JA (Kloek et al., 2001); *npr1*, enhanced susceptibility to pathogens, SA-mediated responses are blocked at NPR (for other functions of NPR, see Spoel et al., 2003); *nahG*, bacterial-SA hydroxylase converts SA immediately to inactive catechol, therefore, SA-mediated responses blocked (Delaney et al., 1994); and *etr1*, insensitive to ET because the ET receptor ETR1 is disabled (Gamble et al., 1998). Identities of *coi1-1* plants were confirmed by the failure of a growth medium containing 50 μM methyl JA to inhibit root growth.

We confirmed that the plant genotypes differed in signal pathway activity by assessing expression levels of marker genes in uninfested control plants of each genotype, compared with the housekeeping gene AC8 (data not shown). No reverse transcription (RT)-PCR product for *PR1* was observed in the genotypes with disrupted SA signaling (*npr1* and *nahG*) as expected. There was little expression of *PR1* in Col-0, but *PR1* was expressed in *hrl1*, *coi1*, and *etr1*, as expected. The RT-PCR product for *BGL1*, a marker for JA pathway activity, was found in all mutants, but the expression level was lower as expected in the JA-insensitive mutant *coi1*. The RT-PCR product for the ET marker *CaEF* was nearly below the detection limit in the ET-insensitive mutant *etr1*, as expected, and in *hrl1*.

Arabidopsis seeds were vernalized and sown into 6- $\times$ 5-cm pots filled with sterile Metromix 200 (Scotts, Marysville, OH; contains sphagnum, peat moss, and horticultural perlite). Plants were kept in growth chambers at 22°C $\pm$ 1°C, 65% $\pm$ 5% relative humidity, at 250 μmol m^{-2} s^{-1} light intensity, and on a 10:14 (light:dark) photoperiod. The photoperiod was changed after 2 weeks for the second experiment with aphids and caterpillars to 8:16 (light:dark) photoperiod, preventing bolting of the *hrl1* mutants. Plants were watered as needed (approximately twice a week) and fertilized every 2 weeks (21-7-7; Miracle Gro, Scotts, Marysville, OH).

Insect Rearing

For bioassays with phloem-feeding insects, we used the specialist aphid *Brevicoryne brassicae* and the generalist *Myzus persicae* Sulzer. As a chewing insect, we used the generalist caterpillar *Spodoptera exigua* (Hübner).

Aphid clones were maintained on pak-choi plants (*Brassica campestris* L. subsp. *chinensis* var Black Behi) at 22°C to 26°C and 12-h photoperiod (50 μmol m^{-2} s^{-1}). New plants were added biweekly, and old plants were removed after aphids settled on the new plants. Electron microscopy was used to determine that aphids were free of insect and plant viruses, which can affect aphid reproduction or may induce plant defenses.

Eggs of *S. exigua* (Hübner) were obtained from Benzon Research (Carlisle, PA). The larvae were grown on commercially available artificial Spodoptera diet from Bioserv (Frenchtown, NJ) until the fourth instar at temperature of 22°C to 26°C. The artificial diet was replaced about every 3 d. One day before the experiment, *S. exigua* were transferred to Col wild-type plants.

Experimental Procedures

We conducted two experiments with two classes of feeding herbivores to study plant responses to insect feeding and insect performance on Arabidopsis with disrupted signaling pathways. Plants were 32 d old for the experiments with *M. persicae* and *B. brassicae* and 36 d old in the experiments with *S. exigua*. There were 10 replicates per genotype in the experiment with aphids and eight replicates in the experiment with *S. exigua*.

Insect bioassays were conducted in cages of transparent mylar cylinders (5 cm diameter, 9 cm high) with a top of fine mesh gauze (mesh width: $<$0.1 mm) and the lower cage edge in the soil. These cages contained the insect but maintained air exchange and allowed the insects to choose their feeding sites.

In the aphid experiments, 10 apterous aphids (adults and fourth nymph stage) were transferred to each of 10 plants of each genotype. Plants with aphids and aphid-free control plants (also caged) were kept in a growth chamber at 22°C ± 1°C, at 200 μmol m^{-2} s^{-1} light intensity, and a 12:12-h photoperiod. After 7 d, the control plants were cut directly above the root and immediately flash frozen in liquid nitrogen. In aphid treatments, the aphids were removed with a brush and counted before the plants were frozen in liquid nitrogen. All material was 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.

In the experiments with *S. exigua*, single fourth-instar larvae were weighed and transferred to each of the eight plants of each genotype. Plants with *S. exigua* and control plants (also caged) were kept in a growth chamber at 22°C ± 1°C, at 250 μmol m^{-2} s^{-1} light intensity, and a 10:14-h photoperiod. Larvae were allowed to feed for 24 h and then removed and reweighed. The leaf area damage was estimated using the categories described by Stotz et al. (2000). One day later (to obtain optimal GS induction), the plants were harvested as described for the aphid experiments; tissues were kept at −80°C for gene expression analysis and six plants (three pairs) were used for GS analysis.

The statistical significance of variation in insect performance among different mutants in each experiment was determined using ANOVA, followed by the posthoc test Tukey's honestly significant difference (HSD) mean-separation test in SAS 8.0 (SAS Institute, Cary, NC) and SYSTAT 10.0 (SPSS, Chicago).

Treatment with JA

A separate experiment was conducted to compare the elicitation of GS production by insect feeding with direct application of JA. This experiment was done with Col-0 plants cultivated under a 12-h photoperiod, at 150 μmol m^{-2} s^{-1}, and at 23°C ± 1°C day and 21°C ± 1°C night temperature, respectively. Five-week-old plants were treated with (±)-1a,2b-3-Oxo-2-(cis-2-pentenyl)cyclopentaneacetic acid (JA; Sigma-Aldrich, St. Louis; 2.5 mM in 1.5% [v/v] ethanol and 0.125% [v/v] Triton in water) or solvent control lacking JA. Treatments were applied as a fine mist to completely wet the adaxial side of rosette leaves on days 1, 2, and 5. After 1 week, plant rosettes were harvested, flash frozen in liquid nitrogen, and stored at −80°C.

GS Extraction and Analysis

GS were extracted three times from lyophilized tissue (20–100 mg) for 5 min with 1 or 5 mL of 70% (v/v) boiling methanol. Extracts were centrifuged at 1,200*g* and supernatants were combined. 4-Hydroxybenzyl-GS, sinalbin (40 or 200 μL of 3 μmol mL^{-1} solution; The Royal Veterinary and Agricultural University, Copenhagen), was added to the extract as the internal standard. Combined supernatants were evaporated almost to dryness at 45°C and were redissolved up to a volume of 2 or 10 mL double-distilled water containing 0.4 M barium acetate to precipitate proteins. After 1 h at room temperature, extracts were centrifuged at 2,600*g* for 20 min. One-half of each supernatant was hydrolyzed overnight with 0.1 unit of myrosinase (Sigma-Aldrich) at room temperature. Hydrolyzed and unhydrolyzed (containing GS) extracts were desulfated on DEAE Sephadex A-25 mini columns in 2 M acetic acid. Before loading GS extracts, columns were rinsed with 2 mL of 6 M imidazole-formate solution in 30% formic acid followed by two washes with 1 mL of dd water. Then columns were washed twice with 1 mL of 0.02 M sodium acetate buffer, pH 4.0. A total of 150 μL of aryl sulfatase solution (Sigma-Aldrich; H-1 from *Helix pomatia*, prepared according to Graser et al. [2001]), was added. Capped columns were incubated overnight and desulfo-GS were eluted with 1 mL of dd water.

Desulfated extracts were separated by HPLC (Waters WISP 710 B, Milford, MA) fitted with a C-18 reverse-phase column (Spherisorb ODS-2, 5 μm, 4.6 × 250 mm; Sigma-Aldrich) using (A) dd water and (B) 20% acetonitrile (HPLC grade in dd water) gradient at a flow rate of 1.5 mL^{-1}. The 39-min run consisted of 1% (v/v) B (1 min), 1% to 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 diode array detection between 190 and 360 nm. GS peaks were identified using retention time and UV spectra. Quantification was done by subtraction of hydrolyzed from and unhydrolyzed extracts at A_{229} as described by Mewis et al. (2002) relative to the internal standard. To calculate molar concentration of individual GS, relative response factors (Buchner, 1987; Brown et al., 2003) were used to correct for absorbance difference between the reference standard (4-hydroxybenzyl-GS, response factor 0.51) and other compounds. GS identities were confirmed by liquid chromatography-mass spectrometry (Perseptive Voyager DESTR MALDI-TOF system).

For each experiment, statistical differences in single GS classes and total GS content among mutants and between treatments were detected by ANOVA and the posthoc Tukey's HSD test in SYSTAT 10.0. To determine whether GS might be responsible for differences among plant genotypes in plant resistance or insect performance, simple Pearson product-moment correlation coefficients were calculated between GS contents and insect performance and consumption results for the same plants. While multiple regression would be an ideal way to examine the relative contribution of specific GS structures to insect performance, we are unable to do so because of the need to combine replicate plants for chemical analyses. For the correlation analysis reported here, the average performance data for a pair of plants is plotted against their combined GS content.

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