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Specificity of Phenolic Glycoside Induction in Willow Seedlings (*Salix sericea*) in Response to Herbivory

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Abstract *Salix sericea* (Marsh.) (Salicaceae) seedlings were used to investigate phytochemical induction of phenolic glycosides following beetle herbivory. Seven-week-old full-sibling seedlings were subjected to one of three damage treatments: *Plagiodera versicolora* adults, *P. versicolora* larvae, or *Calligrapha multipunctata bigsbyana* adults. Salicylate concentrations were measured locally (within damaged leaves) and systemically (above and below damaged leaves) 4 d later. Herbivory caused differential salicylate induction; 2′-cinnamoylsalicortin was induced, whereas salicortin was not. The induction of 2′-cinnamoylsalicortin was not specific with regard to the species or developmental stage of beetle tested but did vary with leaf age: induction occurred in the younger undamaged leaves but not in the damaged leaves or in the older undamaged leaves. The amount of leaf area consumed had no detectable effect on induction, indicating an "all-or-none" response triggered by even small amounts of herbivory. Locally, herbivory caused a decrease in salicortin concentrations, probably because of degradation within the damaged leaves. These results suggest a specific but generalized induced response to these leaf-feeding beetles.

Keywords Herbivory · Chemical induction · Specificity of elicitation · *Salix* · *Plagiodera versicolora* · *Calligrapha multipunctata bigsbyana* · Phenolic glycoside · Salicylate

Introduction

Plants contain a wide variety of secondary metabolites that function as defenses against herbivores. Many of these compounds are inducible, and if the response is specific, it may limit resource allocation and ecological costs (Karban and Baldwin, 1997; Agrawal and Karban, 1999). Specificity of induction to herbivory can be described in two ways: specificity of elicitation (the ability of the plant to generate distinct chemical responses to

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different herbivores) and specificity of effect (the range of organisms affected by a given induced response) (Karban and Baldwin, 1997; Stout et al., 1998; Agrawal, 2000). Specificity of elicitation, the focus of our study, occurs when the induced response varies among herbivores, across chemical types, or if it varies in magnitude or location within the plant. For example, mechanical wounding can differ from herbivory (Hartley and Lawton, 1987; Turlings et al., 1990; Mattiacci et al., 1995; Alborn et al., 1997; McCloud and Baldwin, 1997), and different herbivores, species and/or developmental stages, can generate distinct responses (Stout et al., 1994; Takabayashi and Dicke, 1996; Inbar et al., 1999; Schittko et al., 2000). Furthermore, certain compounds within a class may be induced and not others (Clausen et al., 1989; Bodnaryk, 1994; Stout et al., 1994, 1998; Doughty et al., 1995; Ruuhola et al., 2001), or the response may only occur in certain tissues (Jones et al., 1993; Stout et al., 1996). Whereas these studies document specificity of induction, no single study simultaneously examines how contrasting herbivores differentially induce closely related chemicals and how these induced responses vary both locally (in the damaged tissue) and systemically (but see combined studies of Stout et al., 1994, 1996, 1998). This study focused on the specificity of phytochemical induction in *Salix sericea*.

Like many members of the Salicaceae, *S. sericea* is attacked by a diversity of herbivores and produces phenolic glycosides, a group of chemicals that deter feeding of many generalist herbivores (Tahvanainen et al., 1985; Lindroth et al., 1988). The two most common phenolic glycosides in *S. sericea* are salicortin and 2′-cinnamoylsalicortin, which can account for 11.6 and 1.5% of the dry leaf mass, respectively (Orians et al., 1996). Induction of phenolic glycosides has been previously reported in cuttings from adult plants of *S. myrsinifolia* but not *S. pentandra* (Julkunen-Tiitto et al., 1995; Ruuhola et al., 2001). More specifically, Ruuhola et al. (2001) only found significant induction of certain phenolic glycosides in undamaged immature leaves, but not in damaged mature leaves of *S. myrsinifolia*. Mature damaged leaves only showed increases in degradation products. Stevens and Lindroth (2005), using 2-yr-old *Populus tremuloides* obtained from root micropropagation, did not observe rapid induction of phenolic glycosides immediately after manual damage but found that levels were elevated later in the season (termed within-season-delayed systemic induction). Although these studies show that induction can occur, it is not known whether different herbivores have contrasting effects or if seedlings can be induced, locally or systemically.

Here, we examined whether *Plagiodera versicolora* adults, *P. versicolora* larvae, and *Calligrapha multipunctata bigsbyana* adults (Chrysomelidae, Coleoptera) induce phenolic glycosides both locally (damaged leaves) and systemically (in undamaged leaves above and below the damaged leaves). We hypothesized that the induction of phenolic glycosides would be pronounced in response to both species, as high concentrations of phenolic glycosides are known to inhibit their feeding and growth rates (Tahvanainen et al., 1985; Orians et al., 1997). We also expected induction to be greatest in the young damaged leaves, intermediate in the younger undamaged leaves, and minimal in mature undamaged leaves.

Methods and Materials

Plants and Insects

Salix sericea (Marshall), silky willow, is a 0.5- to 4-m high shrub that occurs in riparian and swampy areas in eastern Canada and northeastern United States. In New York, *S. sericea* produces leaves continuously from May until September. Plants flower in late April, and

the female plants set seed at the end of May. Seed germination occurs immediately. *S. sericea* is attacked by numerous specialist and generalist herbivores, including several species of coleopteran leaf chewers (Orians and Fritz, 1996; Orians et al., 1997).

S. sericea full-sibling progeny (S47×S16) was generated from a wild population of willows in Milford, NY. Crosses were made by transferring pollen from S47 male to catkins of S16 female. We used a single full-sib family to minimize possible genetic variation in constitutive chemistry and induced responses (Stevens and Lindroth, 2005). Catkins had been covered with mesh pollination bags to prevent visitation by insect pollinators, and, once pollinated, bags were replaced and left until seed maturation. On June 3, 2002, seeds were sown in separate trays, and the resulting seedlings were grown in a shaded open-end greenhouse (30% of full sun) located at our field station in Milford, NY. Following germination, each seedling was transferred to a 0.75-dl pot. Seedlings were watered daily and received a weekly standard solution of fertilizer (6 g/l of Peters Professional NPK 20:20:20). The experiments were initiated on July 28, when the seedlings were between 6 and 13 cm tall and had 5–11 fully expanded leaves.

Beetles (Coleoptera: Chrysomelidae) were collected from the immediate area surrounding the field station on the day before the experiments were initiated. *C. multipunctata bigsbyana* (Kirby) adults and *P. versicolora* (Laicharting) adults and larvae, hereafter referred to as *Calligrapha* adults and *Plagiodera* adults and larvae, respectively, were collected while feeding on *S. sericea* and *S. sericea* × *S. eriocephala* hybrids. While both species are generalist feeders on *Salix*, *Calligrapha* is native, whereas *Plagiodera* is not. It is, therefore, conceivable that the response to the native *Calligrapha* could be greater. Adults were held without food prior to induction assays; larvae were caged along with the leaf they were feeding on at the time of collection.

Plant Treatments

Treatments were initiated on July 28, 2002 (d 0). Each seedling was randomly assigned to one of four treatments: *Plagiodera* larvae (PVL; N=28), *Plagiodera* adults (PVA; N=29), *Calligrapha* adults (CMA; N=30), and control (CON; N=26). Each seedling was randomly placed within the greenhouse on d 0 and again on d 2.

For each plant, a clip cage (5.0-cm diameter, consisting of two plastic petri plate tops hinged together with a metal hair clip and ventilated with eight 1-mm holes) was attached to the third youngest fully expanded leaf on d 0, ensuring that the entire leaf was inside the cage. Preliminary experiments on *S. sericea* found that the clip cages had no effect on salicylate concentrations after 4 d of being attached to the plant (unpublished data). Plants in the PVL group received a clip cage containing two *Plagiodera* larvae, plants in the PVA group received a clip cage containing two *Plagiodera* adults, plants in the CMA group received a clip cage containing one *Calligrapha* adult, and plants in the CON group received an empty clip cage. Two *Plagiodera* were used because a single *Calligrapha* adult consumes more leaf material per unit time. On d 1, clip cages were transferred to the next youngest, fully expanded leaf. On d 2, all clip cages and herbivores were removed from the plants.

Based on previous experiments looking at induction strength over time, plants were harvested on d 4, the time of maximal induction (unpublished data). Foliage was divided three ways: local, systemic young (SY), and systemic mature (SM) leaves. The local sample consisted of the two leaves enclosed within the clip cage, the SY sample consisted of all leaves above (younger than) the locally damaged leaves, and the SM sample consisted of all the leaves below (older than) the local leaves. All leaves from each location (SY, local, and

SM) were vacuum-dried at room temperature for 2 d, weighed, and stored at −20°C until high-performance liquid chromatography (HPLC) analysis.

Chemical Analysis

Salicortin and 2′-cinnamoylsalicortin were analyzed with standard techniques (Orians 1995). Briefly, leaf samples were ground to a fine powder and mixed thoroughly using a ball-mill grinder (Kleco). For each sample, 10.0±0.3 mg leaf powder, or as much tissue that was available, were added to a microcentrifuge tube. Each tube received 1.0 ml of 6.4 mM 1,3-dimethoxybenzene in methanol as an internal standard. Following 5 sec of vortexing, the samples were sonicated at 1–2°C for 13 min, and centrifuged at 7,000 rpm/min for 3 min. The extract was poured off and filtered using 0.45-µm pore Acrodiscs. Samples were analyzed at 277 nm on an HPLC (Hewlett Packard Model 1100) equipped with a 3.9× 150 mm Nova-Pak C18 column (Waters) and an UV detector, using a gradient of methanol and dH_2O. Peaks representing phenolic glycosides were quantified using purified laboratory standards.

Measurement of Leaf Damage

Because the amount of leaf area consumed varied among herbivory treatments, we tested whether the amount of leaf area consumed had an effect on chemical concentrations. To estimate leaf area, leaves were photocopied and their images scanned by using NIH Imaging Software version 1.62 (http://rsb.info.nih.gov/nih-image/). For those plants that received herbivory, this was a measurement of the postherbivory area. To estimate preherbivory leaf area, leaves were photocopied and the missing portions of the chewed leaves were filled in. The manipulated image was scanned and analyzed as before to estimate preherbivory leaf area. The amount of leaf area consumed was calculated as: (preherbivory area) − (postherbivory area). If a leaf was consumed entirely, its area was assumed to be equal to the area of the plant's other local leaf.

Data Evaluation and Statistics

The amount of leaf area consumed was compared among the treatments using one-way ANOVA, and correlations were performed to determine if the amount of leaf area consumed was related to phenolic glycoside concentrations. Because larger seedlings (as measured by total above-ground biomass) had higher concentrations of phenolic glycosides (data not shown), we also examined the correlation between the total mass of the leaves at each location (local, SY, or SM leaves) and 2′-cinnamoylsalicortin and salicortin concentrations. There was a strong positive correlation for all samples (see Results), further indicating that larger plants produced higher concentrations of phenolic glycosides. Total leaf mass at each location was, therefore, included as a covariate in subsequent analyses to control for the effects of seedling size.

To examine the effects of leaf age on baseline phenolic glycoside concentration, we compared the concentration of the two phenolic glycosides across the three leaf locations (SY, local, and SM) in CON plants using a one-way analysis of covariance (ANCOVA; with location as the main effect and total leaf mass as the covariate). To test for induction at each leaf location, the effect of herbivore treatment was analyzed by using a one-way ANCOVA (with herbivore treatment as the main effect and leaf tissue mass as the covariate). Significance of differences among leaf locations and treatments were determined

with a LSMeans Student's *t*-test (JMP statistical software version 5.0.1.2, SAS Institute Inc., Cary, NC, USA, 1989–2003).

Results

2′-Cinnamoylsalicortin and salicortin were detected in all samples and accounted for 1.30±0.09 and 11.59±0.30% (mean±SE), respectively, of the mean dry leaf mass of CON plants (similar to previous reports on this species [Orians et al., 1996]). There was a strong positive relationship between 2′-cinnamoylsalicortin and salicortin concentrations within SY, local, and SM leaves for all treatments (r^2=0.366, 0.414, and 0.153, respectively; $P<0.001$). There were also strong positive relationships between total leaf mass and salicylate concentrations within the SY, local, and SM leaves for all treatments (for 2′-cinnamoylsalicortin, r^2=0.249, 0.260, and 0.322, respectively, and for salicortin, r^2=0.283, 0.264, and 0.146, respectively; $P<0.001$). The same trend was observed in the local leaves of the CON treatment when analyzed separately, indicating that larger plants (more leaf biomass at each location) were capable of producing higher concentrations of phenolic glycosides.

In CON plants, 2′-cinnamoylsalicortin concentrations were highest in the SY leaves, whereas salicortin concentrations were highest in the local leaves (Fig. 1). Specifically, 2′-cinnamoylsalicortin concentrations were significantly higher in SY leaves compared to local leaves and in the local leaves compared to the SM leaves (Fig. 1a–c; F=26.01; $P<0.001$). Salicortin concentrations were significantly higher in local leaves compared to SY and SM leaves, whereas SY and SM leaves had similar concentrations (Fig. 1d–f; F=12.03; $P<0.001$).

Plants in the CMA treatment lost the greatest amount of leaf area to herbivory (2.490±0.230 cm^2), whereas plants in the PVL treatment lost the least amount of leaf area to herbivory (1.370±0.200 cm^2), significantly less than the plants in the CMA treatment ($P<0.001$). The amount of leaf area lost to herbivory in the PVA plants (2.110±0.200 cm^2) was not significantly different from either of the other two treatments ($P>0.05$). Leaf area removed by PVL, PVA, or CMA was not positively correlated with phenolic glycoside concentration. In fact, leaf area removed was correlated with lower phenolic glycoside concentration in three cases: for both 2′-cinnamoylsalicortin and salicortin concentration in the local leaves in response to *Plagiodera* larvae, and for salicortin concentration in the SY leaves (Table 1).

Of the two phenolic glycosides, only 2′-cinnamoylsalicortin was induced. There was a significant overall treatment effect of herbivory on 2′-cinnamoylsalicortin concentration in the SY leaves (F=2.71; P=0.049). Mean 2′-cinnamoylsalicortin concentration was higher in SY leaves of the three herbivore treatments than in CON treatment (Fig. 1a), whereas 2′-cinnamoylsalicortin concentration in local and SM leaves were similar among the treatments (Fig. 1b, c). Mean salicortin concentrations in SY and SM leaves were similar among all treatments (Fig. 1d, f). Mean salicortin concentrations were significantly lower in local leaves of the PVL plants than in CON plants (Fig. 1e).

Discussion

Overall, we found evidence for specificity of induction. All herbivores tested caused 2′-cinnamoylsalicortin concentrations to increase systemically, but only in the youngest,

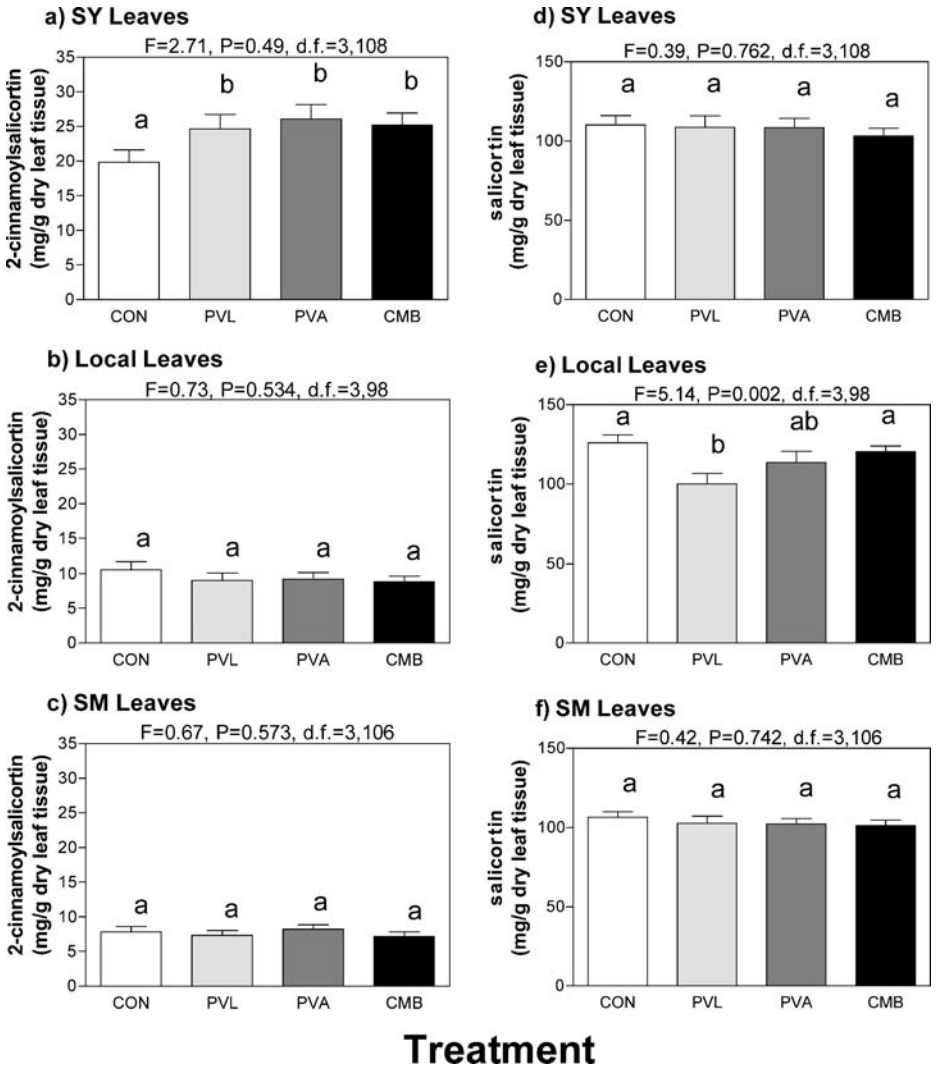

Fig. 1 The effects of treatments on least square mean (±SE) 2′-cinnamoylsalicortin (**a**, **b**, **c**) and salicortin (**d**, **e**, **f**) concentrations in SY, local, and SM *Salix sericea* leaves. Treatments were compared by one-way ANCOVA with leaf tissue mass as a covariate. Different letters above bars indicate significant differences between treatments (P=0.05). ANCOVA results are presented above the graph. SY = systemic young leaves, local = local leaves, SM = systemic mature leaves, CON = clip cage controls (N=26), PVL = *Plagiodera versicolora* larvae (N=28), PVA = *P. versicolora* adults (N=29), CMB = *Calligrapha multipunctata bigsbyana* adults (N=30)

undamaged upper leaves. In contrast, there was no increase in salicortin concentration. Thus, young *S. sericea* seedlings are capable of increasing the concentration of 2′-cinnamoylsalicortin in young leaves in response to beetle herbivory. Whereas the three herbivore treatments caused similar patterns of induction in young undamaged leaves, *P. versicolora* larvae actually caused a significant drop in salicortin levels within damaged leaves.

Table 1 Pearson's correlation coefficients (r) representing the relationship between leaf area consumed by herbivores and the concentrations of 2′-cinnamoylsalicortin and salicortin in SY, local, and SM *Salix sericea* leaves

Herbivore treatment	2′-Cinnamoylsalicortin			Salicortin		
	SY	Local	SM	SY	Local	SM
PVL	−0.293	−0.479**	−0.277	−0.443*	−0.592***	−0.253
PVA	−0.118	−0.134	−0.167	0.261	0.214	0.089
CMA	0.263	−0.155	−0.008	0.110	−0.063	−0.045

SY = Systemic young leaves, SM = systemic mature leaves, PVL = *Plagiodera versicolora* larvae, PVA = *P. versicolora* adults, CMA = *Calligrapha multipunctata bigsbyana* adults.

*$P<0.05$.

**$P<0.01$.

***$P<0.001$.

Patterns of induction are best examined in context of baseline levels of phenolic glycosides. The distribution of phenolic glycosides within the control seedlings varied according to the compound. The distribution of 2′-cinnamoylsalicortin showed a clear negative relationship with leaf age (e.g., the young sink leaves had the highest concentrations, whereas the oldest mature leaves had the lowest). The spatial distribution of salicortin, however, revealed a different pattern: local leaves had significantly higher salicortin concentrations than both systemic young and systemic mature leaves. This result is consistent with previous work on *S. sericea* seedlings (Orians, unpublished data). Similarly, Julkunen-Tiitto et al. (1995) found that whereas the concentrations of many phenolic glycosides are highest in the young shoot tips of *S. myrsinifolia*, the concentration of others (e.g., salicortin-2) are highest in the mature leaves. These results indicate that phenolic glycoside biosynthesis can continue after leaf transition from sink to source leaves (see also Kleiner et al., 1999).

Not only were 2′-cinnamoylsalicortin concentrations highest in youngest leaves, but the three beetle treatments induced significant increases in the concentration of 2′-cinnamoylsalicortin, but not salicortin, in these younger leaves. Ruuhola et al. (2001) also found phenolic glycoside induction to be greatest in leaves with constitutively higher concentrations and suggest that induction of phenolic glycosides was probably a result of increased biosynthesis. It is interesting that 2′-cinnamoylsalicortin, which rarely exceeds 2% dry leaf mass, was induced but that salicortin, which often exceeds 10% dry leaf mass, was not. This could be due to the fact that 2′-cinnamoylsalicortin is most likely synthesized by adding a 2′-cinnamoyl group to salicortin, and, thus, the observed increase may be a result of enhanced conversion of preexisting salicortin to 2′-cinnamoylsalicortin. Because salicortin levels decreased in the locally damaged leaves, it is tempting to speculate that this was the source of salicortin. There is, however, no evidence to date that phenolic glycosides are transported within the vascular system from leaf to leaf. Rather, we suggest that any decrease in salicortin concentrations in SY leaves went undetected because salicortin concentrations were so much higher than 2′-cinnamoylsalicortin concentrations.

We had expected the damaged leaves to exhibit the greatest induction, but none was observed. As expected, induction was absent in the SM leaves. Whereas SY leaves exhibited induction, the lack of 2′-cinnamoylsalicortin induction in damaged leaves suggests that this compound is not inducible in source leaves (see also Ruuhola et al., 2001). Compared to control plants, all three herbivore treatments exhibited lower mean

salicortin concentrations in locally damaged leaves, although the PVL treatment was the only treatment that showed a significant difference. This decrease probably represents chemical degradation (Clausen et al., 1989; Julkunen-Tiitto et al., 1995) and would suggest that 2′-cinnamoylsalicortin is less prone to degradation. Clausen et al. (1989) reported that crushing *P. tremuloides* leaves induced a rapid (within seconds to minutes) enzymatic conversion of salicortin to salicin and 6-HCH. Although few of the samples in this study contained detectable quantities of salicin (personal observation), it is possible that salicin degraded even further in the days following wounding. Differences in local salicortin concentrations between the *Plagiodera* adults and larvae treatments may reflect different feeding strategies. Adult beetles feed by chewing on leaves, from the outer edges inward, whereas larvae feed by skeletonizing the leaves from the inner leaf outward, thereby causing damage to more leaf area per amount consumed and possibly causing more degradation. Alternatively, the lower concentrations found in larva-consumed tissues may reflect the fact that remaining vein tissue does not contain as much salicortin as mesophyl tissue.

Despite differences in the amount of leaf area removed, this did not have an effect on the amount of induction observed, as there were no significant positive relationships between phenolic glycoside concentration and the amount of leaf area consumed by herbivores. These data suggest that induction of 2′-cinnamoylsalicortin in *S. sericea* may be an "all- or-none" response triggered by even small amounts of herbivory by these beetles. In previous experiments, mechanical damage designed to mimic herbivory did not produce an induced response (Fields, unpublished data), indicating that 2′-cinnamoylsalicortin induction in *S. sericea* occurs in response to a specific cue presented by the beetles. Would the same pattern of induction be observed following herbivory by a highly specialized beetle such as *Chrysomela* spp., which use phenolic glycosides as both feeding stimulants and as a source of defensive metabolites? We suggest that such herbivores would not elicit a strong induction of phenolic glycosides in willow because this could result in substantial costs to the plant. Although preliminary results in 2001 with *Chrysomela knabi* support this prediction (personal observation), we were unable to test this in 2002 because *C. knabi* were absent that year.

The ecological consequences of these induced changes are unknown. We suggest that induction could reduce subsequent feeding by herbivores such as *Plagiodera*, which are known to be sensitive to changes in phenolic glycoside concentration (Tahvanainen et al., 1985; Orians et al., 1997). Our data suggest a possible negative effect on beetle feeding: leaves with constitutively higher phenolic glycoside concentrations received significantly less larval *P. versicolora* herbivory. Interestingly, there was no clear pattern or significant differences for adult *P. versicolora*, suggesting that feeding preference may be a function of developmental stage. Although *C. multipunctata bigsbyana* also prefers foliage with moderate to low concentrations of phenolic glycosides (Orians et al., 1997), we found no association between leaf chemistry and leaf area consumed for this species.

One of the main questions raised by this study asks why 2′-cinnamoylsalicortin is inducible but salicortin is not. We suggest three possibilities that merit further investigation. First, salicortin production may be hindered during leaf expansion. Second, salicortin is known to be an oviposition/feeding stimulant for a number of beetles (Tahvanainen et al., 1985; Orians et al., 1997). If 2′-cinnamoylsalicortin is not increasing its concentrations, holding salicortin concentrations steady might represent a strategy to minimize future herbivory and oviposition. Third, plants may induce 2′-cinnamoylsalicortin because it is more bioactive. Although we favor the first explanation, we cannot reject the other two.

Experiments examining the effects of 2′-cinnamoylsalicortin and salicortin on beetle behavior and performance would be useful in assessing the plausibility of the second and third explanations.

In summary, there is accumulating evidence for specificity of induction in terms of which chemicals are induced, the developmental stage of the leaves that are induced, and the agent of damage (Stout et al., 1996; Agrawal, 2000; Ruuhola et al., 2001). For *S. sericea* seedlings, we observed induction of one of two phenolic glycosides but only in young sink leaves. Although all herbivores tested caused similar patterns of induction, the question whether more specialist beetles that can easily detoxify phenolic glycosides cause similar effects requires further study. Interestingly, the magnitude of induction was not influenced by the amount of leaf area consumed or the species/stage of herbivore feeding, indicating an all-or-none response triggered by even small amounts of damage by these beetles.

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