

Manipulation of the phenolic chemistry of willows by gall-inducing sawflies

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The ability to induce galls on plants has evolved independently in many insect orders, but the adaptive significance and evolutionary consequences of gall induction are still largely unknown. We studied these questions by analyzing the concentrations of various plant defense compounds in willow leaves and sawfly galls. We found that the galls are probably nutritionally beneficial for the sawfly larvae, because the concentrations of most defensive phenolics are substantially lower in gall interiors than in leaves. More importantly, changes in chemistry occur in a similar coordinated pattern in all studied willow species, which suggests that the insects control the phenolic biosynthesis in their hosts. The resulting convergence of the chemical properties of the galls both within and between host species indicates that the role of plant chemistry in the evolution of host shifts may be fundamentally less significant in gallers than in other phytophagous insects.

The ability to induce galls on plants has evolved convergently in at least seven insect orders, and in most of these orders there have been multiple independent origins (1, 2). One of the most plausible explanations for this is suggested by the Nutrition Hypothesis (3), which assumes that gallers are able to manipulate their hosts into producing tissue that is nutritionally superior to other plant parts because it contains high amounts of nutrients and/or low concentrations of defensive chemicals. Studies testing the hypothesis have, however, produced ambiguous results; in many cases, the galls have even been found to contain higher concentrations of defense chemicals than normal plant tissues (4).

On the other hand, interpretation of the results has proven to be somewhat problematic. Many studies have analyzed whole galls, although the insects typically feed on only a small fraction of the gall. Furthermore, all studies have been made with low chemical resolution, i.e., they have measured, for example, “total phenolics” instead of individual compounds. Such analyses can theoretically obscure ecologically meaningful patterns (5) or even lead to misleading results (6). Consequently, the purpose of the present study was to gain a detailed view of the chemical ecology and evolution of gallers. For this, we used HPLC to analyze the concentrations of a large number of different phenolic defense compounds in sawfly galls and willow leaves.

Willows (*Salix* spp.) are one of the most taxonomically and ecologically diverse plant genera in the Northern Hemisphere (7). Chemically, they are characterized by phenolic compounds that occur in various species-specific arrays (8). Some of these phenolics presumably have a defensive role, because flavonoids (9), salicylates (10–12), cinnamic acid derivatives (13), and tannins (14) have been shown to act as feeding deterrents, growth inhibitors, and toxins against insect herbivores. The chemical variability of willows is enhanced by the occurrence of many compounds as various sugar conjugates (glycosides) that may have different effects on herbivores (9).

Despite their defenses, willows are used by a wide variety of phytophagous insects, one of the most important groups of which are the nematine sawfly gallers (Hymenoptera: Tenthredinidae). The nematines that induce true closed galls form a monophyletic group derived from species with folivorous larvae (15). Gall formation is initiated by the ovipositing female and, depending

on the species, the feeding larva may stimulate its continued growth (16).

Six monophagous *Pontania* species were included in our study (host plants in parentheses): *P. arcticornis* (*Salix phylicifolia*), *P. myrsiniticola* (*Salix myrsinites*), *P. nivalis* (*Salix glauca*), *P. samolad* (*Salix lapponum*), *P. aestiva* (*Salix borealis*), and *P. reticulatae* (*Salix reticulata*). The gallers are closely related and belong to the monophyletic subgenus *Eupontania* Zinovjev, which includes species that induce pea- or bean-shaped galls close to the leaf midrib (15, 16). In contrast, the host willows are chemically and taxonomically divergent, ranging from the creeping tundra species *S. reticulata* to the tree-like *S. borealis*. We used HPLC to determine the concentrations of 36 phenolic compounds in the gall cortex, the gall interior, the galled leaf, and an ungalled leaf in 10 individuals per host species. In addition, we determined the concentration of condensed tannins by using a colorimetric test.

Materials and Methods

Sample Collection. Samples of willows and galls were collected from within a 3-km radius of the Kilpisjärvi research station (69°3' N, 20°48' E) in Finnish Lapland. The samples were collected in 1997 between 8 and 11 August, i.e., at a time when the galls have reached their maximum size and the larvae are growing fast. A single leaf with a gall was taken from each individual willow, together with an ungalled leaf immediately below the galled one. Each gall was cut open, and the larva was removed. The samples were dried at room temperature and then stored at –20°C. Before the chemical analyses, the cortex and interior of the gall were separated with a scalpel under a preparation microscope. Samples of the leaves were taken with a hole punch.

Chemical Analyses. The tissue samples (1.3–30.6 mg) were weighed, put into 2-ml Eppendorf tubes, and crushed with a glass rod, and then 0.45 ml pure methanol was added to the tube. After further homogenization, they were allowed to stand on ice for 15 min, after which they were homogenized again and then centrifuged (3 min, 16,000 × g). The supernatant was collected, and the residue was reextracted by using 0.45 ml methanol (2 min on ice). The supernatants were combined, and the methanol was evaporated under nitrogen flow. The extract was redissolved in 1 ml methanol, 0.5 ml of which was used for HPLC and 0.1–0.5 ml for a butanol/HCl–colorimetric tannin assay, in which purified tannin from *Salix purpurea* leaves was used as a standard (17, 18). The HPLC sample was evaporated under nitrogen flow and stored at –20°C. Before HPLC, the samples were redissolved in 0.4 ml methanol–H₂O (1:1). The HPLC runs were processed as described (19), except that the autoinjection volume was 20/25 μl and the column oven was set to 30°C.

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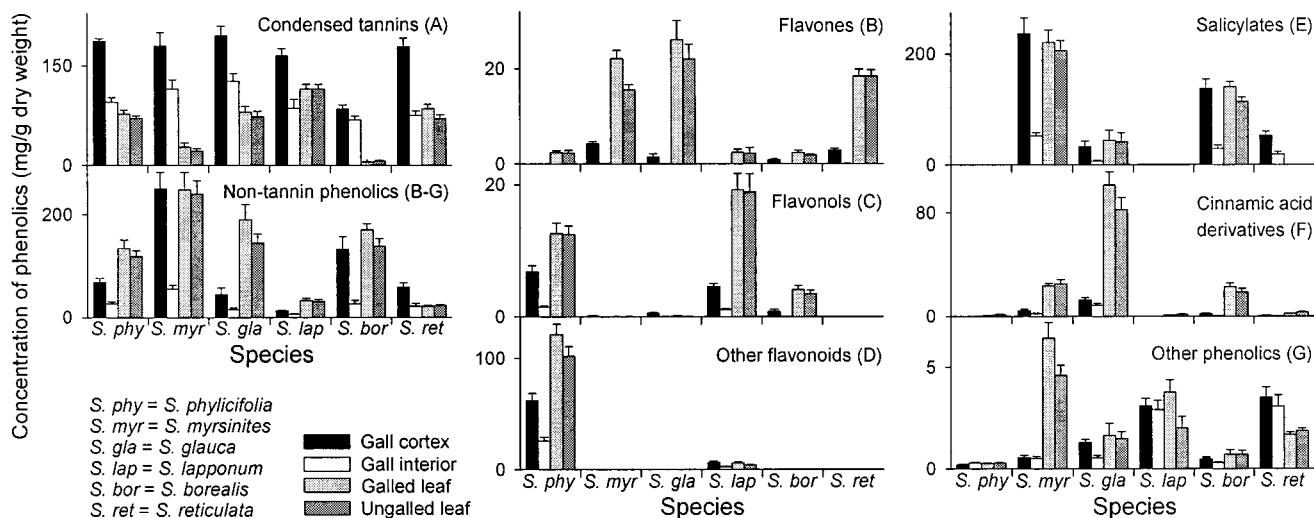


Fig. 1. Mean (+1 SE) total concentrations for different categories of phenolic compounds in the 24 sample classes. Letters in parentheses refer to the chemical categories in Fig. 4 and Table 1 in Supplementary Material (www.pnas.org). Repeated-measures analyses of variance on the totals show that the effect of sample, species, and their interaction, are statistically highly significant in all chemical categories (Table 3 in Supplementary Material).

Peaks were detected at 270 nm, except for chlorogenic acid, which was scored at 360 nm. Only peaks that could be reliably quantified in all species were scored, but the analyzed compounds represent on average >90% of the absorption of all peaks present in the HPLC chromatograms at 270 nm. Quantifications were based on specific standards for the analyzed compounds, with the following exceptions: flavones were quantified as luteolin-7-glucoside or apigenin-7-glucoside, flavonols as myricetin-3-rhamnoside or quercetin-3-galactoside, isorhamnetin-3-glucoside as quercetin-3-galactoside, and ampelopsin derivatives as ampelopsin. Cinnamic acid derivatives were quantified as chlorogenic acid, picein derivatives as picein, and two unknown compounds as salicin.

Statistical Analyses. The mean concentrations ($n = 4-10$) of the analyzed phenolic compounds were calculated for each of the 24 sample classes (Fig. 4 and Table 2 in Supplementary Material; see www.pnas.org). On the basis of their structure, the analyzed compounds can be grouped into different categories (20); the total concentrations of the various chemical categories were calculated for each sample, and the sums were averaged for each sample class (Fig. 1). Samples having any missing data were excluded from the calculation of the mean totals.

General linear model repeated-measures analyses of variance on $\log(x+1)$ -transformed totals were performed by using SPSS version 8.0 (SPSS, Chicago), treating Sample as the within-subjects factor and Species as the between-subjects factor. The degrees of freedom in the within-subjects tests were adjusted by using the Greenhouse-Geisser ϵ coefficient. Repeated-measures-ANOVAs were performed by using the original data (where the sample size in cells varies), and a data set in which any missing data in a given compound was replaced by a randomly selected value from the same sample class before calculation of the total for the sample (Table 3 in Supplementary Material, www.pnas.org). Both methods produced identical results.

Overall chemical similarities were analyzed by using clustering and ordination methods in PC-ORD version 4.01 (MjM, Glenden Beach, OR). In the within-species clustering analyses, inter-sample squared Euclidean distances were calculated from z-transformed (mean = 0, SD = 1) concentrations, which gives an equal weight to all compounds. Only samples having no missing data were used. Clustering analyses were performed by using

UPGMA (Unweighted Pair Group Method Using Arithmetic Averages) complete linkage, and Ward's method.

Between-species analyses were based on z-transformed means of the concentrations of the 37 compounds in each sample class. In the nonmetric multidimensional scaling ordination, ordinal distances were based on Euclidean distances, and 100 replicates with random/PCA starting coordinates were performed. The data were also analyzed by using principal components analysis (PCA; crossproducts matrix calculated by using correlations) and the clustering methods mentioned above. These analyses were performed by using both sample classes and the 177 individual samples that had no missing data.

Results

The chemical properties of the galls differ dramatically from the foliar chemistry of the respective host plants (Fig. 1; see also Fig. 4 and Table 2 in Supplementary Material, www.pnas.org). Individual compounds behave differently, but some changes seem to be highly correlated. For example, gall interiors contain only trace amounts of flavones and flavonols, and the concentrations of salicortin, tremulacin, ampelopsin, and chlorogenic acid are greatly reduced if the host contains these chemicals. In some cases, individual phenolics increase slightly in galls (e.g., triandrin in *S. lapponum* galls), but the increases occur in compounds with low initial concentrations. Interestingly, some phenolics that are virtually absent from host leaves can be found in galls (e.g., salicylates in *S. reticulata* galls), but also in these cases the levels remain low. Overall, gall interiors contain fewer different low molecular-weight phenolics than leaves, and the total concentration of these nontannin compounds is greatly reduced. In contrast, the concentration of condensed tannins is generally higher in gall interiors than in leaves; the highest tannin levels, however, are found in gall cortices (Fig. 1).

The absence of some compounds from the gall interiors combined with the low concentrations of the remaining phenolics leads to a clear reduction in the intraspecific (between-individual) chemical variability of galls in comparison with that of leaves (Fig. 2). In all intraspecific clustering analyses of individual samples, gall samples form a separate cluster, and only some gall cortex samples cluster with leaf samples. The mean and maximum distances between gall interior samples are clearly smaller than those between leaf samples of the same willow

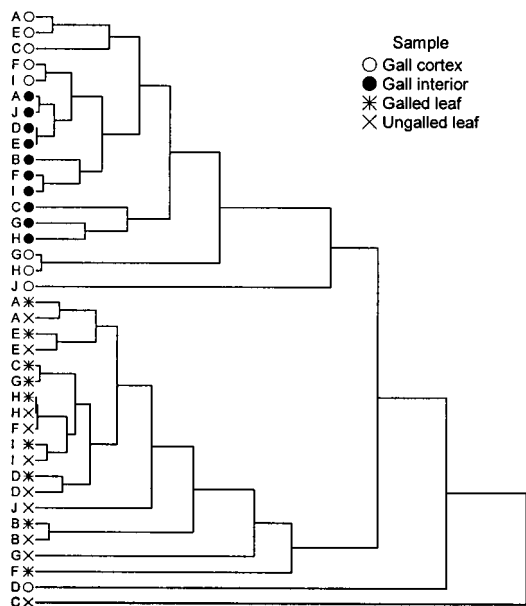


Fig. 2. UPGMA (Unweighted Pair Group Method Using Arithmetic Averages) clustering dendrogram of individual *S. reticulata* gall and leaf samples. Letters denote willow individuals.

individuals, as can be seen in the case of *S. reticulata* (Fig. 2). In the other species, the maximum distance between gall interior samples is 7–36% of the maximum distance between leaf samples (Fig. 5 in Supplementary Material, www.pnas.org).

Similarly, galling markedly reduces between-species chemical variability: the nonmetric multidimensional scaling ordination shows how gall interiors form a tight group, although the host species are chemically divergent (Fig. 3). Downweighting flavonoids by using only the total concentration of the different derivatives of each aglycone (e.g., myricetin glycosides) has no meaningful effect on the ordination results. Essentially similar results were also obtained by using principal components analysis and clustering methods (results not shown). Using individual samples instead of sample classes in the analyses does not affect the outcome: the gall samples form a separate group or cluster, and only some gall cortex samples are grouped with leaf samples. Notably, within the gall sample cluster, gall interior samples are not grouped according to species, i.e., within-species distances exceed between-species distances. Despite this, the maximum

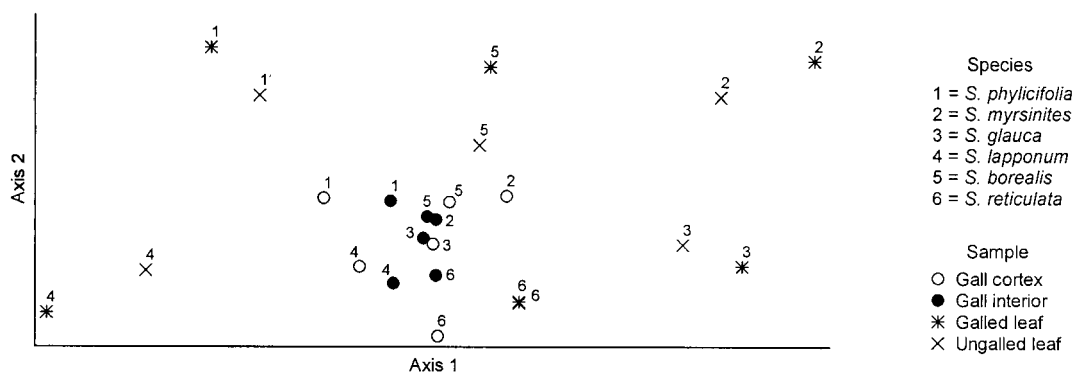


Fig. 3. Nonmetric multidimensional scaling ordination of sample classes. Axes 1 and 2 explain 77.9 and 9.3% of the variance in the original distance matrix, respectively (87.2% combined). Final stress, 9.6%.

distances between gall interior samples are comparable to, or below, within-species distances of the leaf samples.

Discussion

Recent phylogenetic analyses of gall-inducing aphids (21), cynipid wasps (22), thrips (23), and sawflies (15) have shown that the insects, not their host plants, determine the location, size, and shape of galls. Thus, gall morphology can be regarded as an extended phenotype [*sensu* Dawkins (24)] of the galler. Our present results take this conclusion a significant step further, because apparently the insects also control the chemical properties of the galls. As a result, the sawfly galls are able to manipulate their willow hosts into producing large amounts of predictable plant tissue, in which the concentrations of most phenolic compounds are clearly lower than in leaves.

The reduction in the levels of most phenolics certainly fits the prediction of the Nutrition Hypothesis, as does the fact that the concentrations of virtually all analyzed compounds are lower in gall interiors than in gall cortices. The larvae could also benefit from the reduction in the actual number of different phenolics present in the galls, because it may be generally easier for insects to adapt to one or a few toxic chemicals (9, 25, 26). Chemical variability may indeed benefit plants (27), especially as various defensive compounds have been shown to have synergistic effects (9, 10, 26). According to our results, gall induction is an addition to the list of methods (28) by which insects can reduce the chemical variation in their diets.

However, further studies are needed to demonstrate unambiguously that galls are of a higher nutritional quality than other plant tissues. Especially the levels of primary metabolites and inorganic nutrients should be determined in galls and leaves. Furthermore, the reduction in the concentrations of low molecular-weight phenolics in gall interiors is accompanied by an increase in the levels of condensed tannins, and studies on the relative toxicity of these compounds are needed. However, we note that according to Ayres *et al.* (14), condensed tannins may be less harmful to insects than lower molecular-weight compounds.

Although the observed chemical changes could be produced in many ways, the most likely explanation is that the phenolic biosynthesis in willows is redirected by the gall-inducing stimulus. The analyzed compounds are alternative end products of the phenylpropanoid pathway, along which phenolic compounds are produced in plants (20). Consequently, a reduction in the levels of flavones, flavonols, salicylates, and cinnamic acid derivatives could be achieved simply by partially blocking the respective branching points that lead to these categories. The blocking could result from a specific inhibition of the enzymes that

catalyze the production of compounds in the aforementioned categories or from a stimulation of the production of condensed tannins, which would lead to a depletion of intermediate substrates. It is possible that the underlying mechanism is rather simple, because sawfly galls consist of rapidly growing undifferentiated cells (1), and numerous studies have found a tradeoff between growth and the production of defensive compounds in plants (29). However, such a tradeoff cannot alone explain the change in allocation to different phenolic categories.

Elevated concentrations of condensed tannins have also been found in galls induced by cynipid wasps (30), thrips (31), cecidomyiid midges (32), and adelgid aphids (W. J. Mattson, personal communication), but it is not known whether the increase in tannins is accompanied by a reduction in the levels of other compounds. As in the sawfly galls, the tannins in cynipid galls are known to be concentrated in the outer layers, where they may protect the gall from inquilines and fungal attack (30). The abundance of condensed tannins in galls induced by different gallers on taxonomically highly diverse host plants is a pattern that should be further studied. The similarities could result from common physiological responses of plants to gall-inducing stimuli or, conversely, parallel selection pressures on the insects that possibly determine the chemical properties of the galls.

In the sawfly–willow system that we studied, the end result of the chemical changes is a striking convergence of the chemical

properties of galls induced on different willow species. The fact that the intraspecific chemical variability of gall interiors exceeds variability between species is interesting, because chemical differences between plant species probably promote host specificity in phytophagous insects and partly determine the way in which host shifts occur (33–35). Thus, our results indicate that the causes for the strict host specificity in gallers (2) are not linked to the chemical properties of the hosts; the reason may be in the ability to induce galls *per se* or in constraints (34, 36) on the host recognition system. Because gall interior chemistry is not determined by the host plant, it is not surprising that no connection has been found between galler phylogeny and host plant chemistry in the few studies that have addressed the question (15, 37). Indeed, it is likely that the factors determining the probability of host shifts in gallers are fundamentally different from those in other phytophagous insects, and thus studies on gallers can provide valuable new insights into the evolutionary interactions between plants and herbivores.

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