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Original Article

Tissue and toxin-specific divergent evolution in plant defense

Título: Evolución divergente específica de tejido y toxina en defensa de plantas

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Abstract

A major predicted constraint on the evolution of anti-herbivore defense in plants is the nonindependent expression of traits mediating resistance. Since herbivore attack can be highly variable across plant tissues, we hypothesized that correlations in toxin expression within and between plant tissues may limit population differentiation and, thus, plant adaptation. Using full-sib families from two nearby (<1 km) common milkweed (*Asclepias syriaca*) populations, we investigated genetic correlations among 28 distinct cardenolide toxins within and between roots, leaves, and seeds and examined signatures of tissue-specific divergent selection between populations by Q_{ST}–F_{ST} comparisons. The prevalence, direction, and strength of genetic correlations among cardenolides were tissue specific, and concentrations of individual cardenolides were moderately correlated between tissues; nonetheless, the direction and strength of correlations were population specific. Population divergence in the cardenolide chemistry was stronger in roots than in leaves and seeds. Divergent selection on individual cardenolides was tissue and toxin specific, except for a single highly toxic cardenolide (labriformin), that showed divergent selection across all plant tissues. Heterogeneous evolution of cardenolides within and between tissues across populations appears possible due to their highly independent expression. This independence may be common in nature, especially in specialized interactions in which distinct herbivores feed on different plant tissues.

Resumen

Un factor limitante en la evolución de defensas antiherbívoro en plantas es la expresión no independiente en caracteres que median la resistencia. Debido a que el ataque de herbívoros puede ser altamente variable en tejidos de las plantas, hipotetizamos que correlaciones en la expresión de toxinas dentro y entre tejidos de la planta podría limitar la diferenciación entre poblaciones y, por tanto, su adaptación. Utilizando familias de hermanos verdaderos de dos poblaciones cercanas (<1 km) de algodoncillo común (*Asclepias syriaca*), investigamos correlaciones genéticas entre 28 cardenólidos tóxicos dentro y entre raíces, hojas y semillas, y examinamos señales de selección específica de tejido entre poblaciones mediante comparaciones Q_{ST}–F_{ST}. El número, dirección e intensidad de las correlaciones genéticas entre cardenólidos resultaron ser específicas de tejido, y las concentraciones de cardenólidos individuales entre tejidos estuvieron moderadamente correlacionadas, siendo su dirección e intensidad específicas de cada población. Divergencia entre poblaciones en la química multivariante de cardenólidos y en su concentración total, riqueza y polaridad fue mucho más grande en raíces que en hojas y semillas. Se observó selección divergente en cardenólidos individuales específica de tejido y toxina, excepto para un cardenólido altamente tóxico (labriformina) que mostró selección divergente a nivel de planta completa. Evolución heterogénea de cardenólidos parece posible dentro y entre tejidos a lo largo de poblaciones debido a su expresión altamente independiente. Esta independencia podría ser común en la naturaleza, especialmente en interacciones especializadas en las que distintos herbívoros se alimentan de diferentes tejidos de la planta.

Keywords: cardenolides, common milkweed (*Asclepias syriaca*), genetic correlations, plant defense, plant organs, population differentiation

Introduction

An unresolved issue in evolutionary ecology is determining the factors that prevent plants from evolving better defenses against their herbivores. Although defenses can be favored by natural selection (Agrawal et al., 2012a; Züst et al., 2012), they are also costly in terms of resources that could otherwise be allocated to growth and reproduction (Agrawal et al., 2010; Fine et al., 2006; Rausher, 1996; Strauss et al., 2002). Empirical support for such allocation costs of plant resistance has been mixed (e.g., Hahn et al., 2019; Sampedro et al., 2011; Van Dam & Baldwin, 1998), and there is increasing evidence for other types of ecological costs (Strauss et al., 2002) and genetic correlations that may limit the evolution of defensive traits (Agrawal et al., 2002; Wise & Rausher, 2013).

Evolution of plant resistance depends on available genetic variation and the strength and direction of selection exerted by the community of herbivores (Berenbaum & Zangerl, 1992; O´Reilly-Wapstra et al., 2014; Stinchcombe & Rausher, 2002). However, a major limitation is that multiple resistance traits that experience differential selection may not be able to evolve independently (Agrawal 2005; O´Reilly-Wapstra et al., 2014). Indeed, the extent to which defenses against herbivory are genetically correlated has been of long-standing interest to understand the evolutionary dynamics of plant resistance

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(Rausher, 1996; Strauss et al., 2002). Genetic correlations in the expression of chemical defenses may be generated by shared biosynthetic pathways (Berenbaum et al., 1986; Ding et al., 2020). Given that plant tissues are often targeted by distinct herbivores, we hypothesized that genetic correlations among traits could limit responses to selection for tissue-specific defense. Selection for distinct defensive phenotypes between tissues may occur more easily when genetic correlations among traits are scarce within tissues and independent between tissues (Wagner & Altenberg, 1996). Nonetheless, since plant phenotypes are the result of the concerted expression of many traits shaped by selection (Damian et al., 2020; López-Goldar et al., 2020; Simms, 1990), understanding the evolution of defense may benefit from investigating how traits covary genetically in both a multivariate (i.e., genetic integration; Cheverud, 1982] and univariate sense.

Specialized coevolutionary plant–insect interactions are often viewed as a "dead end" in which the evolution of defenses may be constrained due to reduced genetic variation, increased costs of adaptation, and environmental limitations (Forister et al., 2012; Futuyma & Moreno, 1988). Nonetheless, evolution in specialized systems may occur, thanks to standing genetic variation resulting from a variable selection history in space and time (Barrett & Schluter, 2008; Urban et al., 2020). This may especially be the case if multiple herbivores target different plant tissues or are otherwise negatively impacted by distinct plant resistance traits. Conversely, adaptive differentiation may be hampered because of gene flow between populations, which can be especially exacerbated in shorter distances (López-Goldar & Agrawal, 2021; Sexton et al., 2014). Therefore, population differentiation in plant defense expression may be contingent on the relative selection imposed by herbivores that feed on different plant parts, limits due to the genetic architecture of defense expression (Prasad et al., 2012; Züst et al., 2012), and gene flow.

In the milkweed system (*Asclepias* spp.), cardiac glycosides (i.e., cardenolides) have an exclusive defensive function against their physiological target, the Na^+/K^+-ATPase (hereafter "sodium pump") of animals, including the community of specialized milkweed insect herbivores (Agrawal, 2005; Agrawal et al., 2012b, 2022). Cardenolide richness may reach 30 distinct compounds in individual plants, and both compound diversity and concentration are variable among tissues eaten by insects (Lopez-Goldar et al., 2022; Rasmann & Agrawal, 2011). Certain milkweed cardenolides seem to have evolved in response to high herbivore pressure, thanks to a thiazoline ring in their chemical structure that confers greater toxicity to adapted milkweed herbivores (Agrawal et al., 2021, 2022). Within milkweed populations, natural selection has been reported for higher defense investment in response to the herbivore community (Agrawal, 2004, 2005), and patterns of local adaptation among populations have also been documented along environmental gradients (Agrawal et al., 2015, 2022; Hahn et al., 2019; Woods et al., 2012). Nonetheless, whether cardenolide expression is potentially constrained by genetic correlations and whether such constraints may limit genetic differentiation among populations is still unclear.

Here we characterized cardenolides in roots, leaves, and seeds of two nearby (<1 km) populations of common milkweed (*Asclepias syriaca*) to investigate whether tissue-specific defense chemistry differs within and between populations. Although these populations are in similar old-field habitats, we suspected they may be differentiated due to hedgerow barriers between them and differences in the competitive environments, which can strongly impact which herbivores attack milkweed (Agrawal, 2004). Specifically, we tested for (a) phenotypic differences in defense chemistry across tissues and between populations when grown in a common environment, (b) population genetic differentiation based on >900 SNPs, (c) multivariate genetic integration of cardenolides and genetic correlations among individual cardenolides within and between tissues (based on full-sib families), and (d) signatures of tissue-specific divergent selection between populations (measured by Q_{ST}–F_{ST} comparisons). In summary, our goal was to address the extent to which the evolution of cardenolide expression within and between milkweed tissues can be compartmentalized, and generate hypotheses about the ecological mechanisms driving differences in defense compartmentalization between nearby populations.

Materials and methods

Plant material

Common milkweed is a native perennial growing in disturbed habitats across Eastern North America and can reproduce both asexually via underground rhizomes and sexually by flowers. Common milkweed is virtually self-incompatible (<5% selfing) (Kahn & Morse, 1991; Kephart, 1981), and because its seeds are sired by the insertion of a single pollinium into a flower, all seeds from a single fruit pod typically represent a full-sib genetic family (Gold & Shore, 1995). We collected a single fruit pod from each of 15 randomly selected milkweed individuals separated by at least 5 m (to avoid sampling from the same clone) from Ellis Hollow (EH, 42.431707, −76.388960) and Durland Bird Preserve (DBP, 42.435551, −76.396160) populations in 2018, representing 15 full-sib families from each population. We acknowledge that because all plants were grown from seed collected from natural populations, we cannot exclude maternal effects from impacting the phenotypes we measured.

Seeds were surface sterilized by soaking in 10% bleach for 10 min, rinsed, nicked, and stratified for 8 days at 4 °C. Then seeds were germinated at 30 °C in dark. One family from DBP did not germinate. Germinated seedlings were planted in a mix of 25% perlite, 75% Lamberts soil (LM111; Lamberts, Quebec, Canada) in 10-cm plastic pots, and grown in a growth chamber for 4 weeks (14 hr daylight, 27 °C: 23 °C day:night), following a completely randomized design. Plants were fertilized once with slow-release fertilizer (Osmocote Smart-Release, Marysville, OH) 7 days after planting, dilute fertilizer (N:P:K 20:20:20, 120 ppm N [μg/g]) was applied once at day 15, and plants were watered as needed. *Amblyseius cucumeris* mites were applied to prevent thrips infestation/damage.

At the end of the 4-week growth period, roots, stems, and all leaves were collected from each plant, frozen at −80 °C, and freeze-dried. In parallel, we made five groups of randomly chosen seeds from those previously collected in the field from each family and population. Roots, leaves, and seeds were ground to a fine powder on a mixer mill (Retsch, Haan, Germany) using 5-mm steel beads. Freeze-dried, ground root and leaf samples from each individual plant (3–5 plants × 14–15 families × 2 populations = 139 plants) and seed samples from each corresponding family (5 samples × 14–15 families × 2 populations = 145 seed samples) were analyzed for

cardenolide content using high performance liquid chromatography (HPLC) (see below).

For population genetic analyses, we employed 971 established SNPs genotyped by sequencing from EH and DBP randomly collected individuals (40 and 68, respectively) reported in previous work (Boyle et al., 2022) (see also Supplementary Methods S1).

Extraction and HPLC characterization of cardenolides in plant tissues

Cardenolides were extracted from each tissue sample by adding 1 ml of 100% methanol (spiked with 20 µg of hydrocortisone as internal standard) to 50 mg of ground material and 20 FastPrep beads. Samples were extracted by agitation on a FastPrep-24 homogenizer twice for 45 s at 6.5 m/s, and then centrifuged at 14,000 rpm for 12 min. Supernatants were dried down in a vacuum concentrator at 35 °C, resuspended in 250 µl of 16:16:68 (%) methanol:acetonitrile:water (vol:vol:vol), and filtered using 0.45 µm hydrophilic membranes. Prior to methanol extraction, seed tissue samples were defatted by overnight extraction with 1 ml of hexane, which was then discarded.

We detected and quantified the cardenolides in plant tissues using an Agilent 1100 HPLC with diode array detector and a Gemini C18 reversed-phase column (3 µm, 150 mm × 4.6 mm column, Phenomenex, Torrance, CA). We injected 15 µl of each sample into the HPLC running at a constant flow of 0.7 m/min with a gradient of acetonitrile and water as follows: 0–2 min at 16% acetonitrile; 2–25 min from 16% to 70%; 25–30 min from 70% to 95%; 30–35 min at 95%; followed by 10 min of reconditioning at 16% acetonitrile. Peaks were recorded at 218 nm, and absorbance spectra were measured between 200 and 400 nm. Peaks showing a characteristic single absorption maximum between 214 and 222 nm correspond to the unsaturated lactone indicative of cardenolides. The internal standard (hydrocortisone) showed a single absorption maximum of 241 nm. Individual cardenolide concentrations in each tissue sample were estimated by using the peak area and known concentration of the internal standard on a dry mass basis of plant tissue (µg/mg d.w.). We calculated three estimates of total defenses (hereafter "composite cardenolide variables"): the number of distinct cardenolide peaks (hereafter "cardenolide richness"), the total concentration of cardenolides (hereafter "total cardenolides"), and "polarity index" following Rasmann and Agrawal (2011) for each tissue sample. Briefly, the polarity index was constructed using $P = \text{sum}(p_i T_i)$, where T_i is the retention time of the ith cardenolide weighted by its proportion (p_i) within a tissue. Higher polarity values represent a greater proportion of less polar compounds, which, in general, are related to greater toxicity of cardenolides against milkweed–herbivore specialists (Agrawal et al., 2021, 2022; Lopez-Goldar et al., 2022).

Putative identity of individual cardenolides was assigned after comparing HPLC-UV output obtained from similar methods with LC–MS and NMR analyses following Agrawal et al. (2022).

Statistical analyses

We compared plant cardenolide investment between plant tissues (roots, leaves, and seeds, and whole plant) using a linear mixed effects model in SAS v9.4. Concentrations of individual and total cardenolides, cardenolide richness, and polarity index were analyzed independently as response variables and population (Pop), plant tissue (T), and their interaction (Pop×T) were included as fixed effects. Family (nested within population, F(Pop)) and tissue by family interaction (T×F(Pop)) were included as random factors, and their significance was tested using the likelihood ratio test (Littell et al., 2006). Plant tissue (T) was included as a repeated measure to account for the nonindependence of cardenolide concentrations in root and leaf tissues from the same plant, which was considered as subject. Seeds were considered independent from root and leaf tissue of plant individuals since they were ground from randomly pooled seeds. Overall cardenolide investment in the whole plant at the population level was inferred from factor Pop for each variable. Given that fourteen individual cardenolides were present only in one tissue, only the factors Pop (fixed) and F(Pop) (random) were considered for univariate analyses of each of these variables. Best linear unbiased predictors (BLUPs) were obtained from random effects and used to examine familial genetic correlations between cardenolides within and between tissues (see below). Variance components were estimated by restricted maximum likelihood running again the models from above for each trait considering all factors as random, and the contributions to total variance of F(Pop) and T×F(Pop) were estimated if they were included in the model after the likelihood ratio test. Variables were log-, square or cube root-transformed when appropriate to meet assumptions of normality (Supplementary Table S1). Heterogeneous variance for factor Pop was accounted for in the model for each variable when appropriate because it improved model fit (Supplementary Table S1). In order to analyze all the variables without inflating Type I error due to multiple tests, p-value adjustments were performed using the false discovery rate (FDR) (Benjamini & Hochberg, 1995).

To evaluate the differences in multivariate cardenolide chemistry (hereafter "cardenolome") between plant tissues, we conducted a multivariate analysis of variance (MANOVA) in SAS v9.4, including the concentration of all 28 individual cardenolides as response variables. Pop, T, and their interaction were considered fixed effects, and F(Pop) and T×F(Pop) were considered random effects. We also conducted non-metric multidimensional scaling in SAS v9.4 to spatially visualize the differences in the cardenolome between tissues and populations. Briefly, data were log-transformed and normalized by the standard deviation, and chemical composition of all tissues was analyzed based on Euclidean distances.

Genetic correlations and integration of cardenolides

We examined constraints in cardenolide expression by performing univariate Pearson genetic correlations between individual cardenolides within each tissue using the family BLUPs. Given the large number of correlations performed in this way, p-values were adjusted using FDR as above for each tissue in each population. We also performed familial Pearson correlations between tissues for each individual cardenolide expressed in more than one tissue using family BLUPs for each population. Since the presence of a given compound in one tissue and the absence in another may also suggest constraints in cardenolide expression, we also performed Spearman rank genetic correlations between tissues for each individual cardenolide expressed in more than one tissue, considering undetected compounds in one of the tissues in the family BLUPs as zeroes before computing the correlation. To reduce biases in the correlation estimates due to low

sample sizes, we employed the jackknife procedure of Roff and Preziosi (1994) in all correlations before FDR correction. Finally, we compared the correlation matrices of cardenolides between tissues within the same population and between populations for each tissue by conducting 10,000 Monte Carlo permutations of individual correlation values in SAS v9.4 following Piepho (2005).

To investigate the extent to which cardenolides are genetically integrated between tissues and populations, we followed a standard method commonly applied for multivariate data that corrects for varying sample sizes (Junker et al., 2018; Wagner, 1984). We calculated eigenvalues of the resulting Pearson correlation matrix between individual cardenolide concentrations for each tissue and population at the family level. Since the variance of the eigenvalues represents the integration index obtained at the family level (N = 14–15 familial eigenvalue variances for each tissue and population), it is a measure of the strength of the genetic correlations between cardenolides in a multivariate sense (i.e., genetic integration). To account for different sample sizes between tissues for a given family within population, its genetic integration index was corrected by subtracting the expected value under the null assumption of random covariation between cardenolides ([number of cardenolides for a given tissue within the family – 1]/number of individuals in the family) (Wagner, 1984). We also estimated the genetic integration index at the whole plant level for each family and population using the entire data set of cardenolide concentrations across the three tissues. We then compared genetic integration indices (root, leaf, seed, and whole plant) and populations using a linear effects model in SAS v9.4. Genetic integration index was the response variable, and Pop, T, and their interaction (Pop×T) were included as fixed effects. Plant tissue (T) was included as a repeated measure to account for the nonindependence of root, leaf, and seed tissues and whole plant level from the same family for the comparisons.

Population differentiation and Q_{ST}–F_{ST} comparisons

Population neutral differentiation, F_{ST}, was estimated using 971 neutral SNPs obtained from 108 randomly sampled individuals from EH (40) and DBP (68) milkweed populations (SNP details in Boyle et al., 2022). Wright's F-statistic F_{ST} and confidence intervals were estimated by 1,000 bootstrap simulations with resampling over loci using the program GDA (Lewis & Zaykin, 2001). In order to explore the neutral differentiation among nearby populations, population pairwise F_{ST} was estimated with Arlequin v3.5 (Excoffier & Lischer, 2010). Significance (α = 0.05) of the genetic distances was tested by permuting the individuals between the populations 1,000 times. We also conducted a hierarchical analysis of molecular variance (AMOVA) between and within populations with 50,000 permutations with Arlequin v3.5 to test the null hypothesis of no molecular variation at the population level.

To evaluate whether directional or stabilizing selection might be contributing to the tissue-specific differentiation in the expression of milkweed defenses among milkweed populations, we conducted univariate and multivariate Q_{ST}–F_{ST} comparisons for each tissue and at the whole plant level. Univariate Q_{ST} was estimated for each composite cardenolide variable (total cardenolide concentration, cardenolide richness, and polarity) and individual cardenolide concentrations within each tissue (including whole plant level) as the ratio between the amount of genetic variance among populations (σ^2_{Pop}) and the total genetic variance of the trait ($\sigma^2_{Pop} + 2{\cdot}\sigma^2_A$ in diploid organisms, with σ^2_A as the additive genetic variance: $\sigma^2_A = 2{\cdot}\sigma^2_{F(Pop)}$ for full-sibs; Wright, 1951) by using PROC MIXED in SAS v9.4, considering Pop and F(Pop) as random effects. Q_{ST}–F_{ST} comparisons were conducted by parametric bootstrap with 10,000 simulations in SAS, following the method of Whitlock and Guillaume (2009). This method predicts a null distribution for the Q_{ST}–F_{ST} difference (for F_{ST} estimation, see main text, "Population differentiation and Q_{ST}–F_{ST} comparisons") under the null hypothesis that both the quantitative trait and neutral markers show neutral differentiation (i.e., the Q_{ST} equals the F_{ST}). Traits with significantly higher Q_{ST} than F_{ST} are inferred to be under spatially heterogeneous divergent selection, while $Q_{ST} < F_{ST}$ would be indicative of stabilizing selection, and $Q_{ST} = F_{ST}$ would reflect neutral evolution of the trait (O'Hara & Merila, 2005; Whitlock, 2008). Departures from the null hypothesis of neutral differentiation were tested by examining whether the observed Q_{ST}–F_{ST} difference is in the tail of the neutral Q_{ST}–F_{ST} null distribution. An observed Q_{ST}–F_{ST} difference in the lower tail is indicative of spatially stabilizing selection, while a Q_{ST}–F_{ST} difference in the upper tail is taken as evidence of spatially divergent selection on the trait.

A multivariate Q_{ST}–F_{ST} test was performed to compare the tissue-specific (including whole plant level, as above) genetic differentiation of populations at multiple-trait level using the 28 individual cardenolides under the null hypothesis of $Q_{ST} = F_{ST}$ (Leinonen et al., 2013; Martin et al., 2008). This test was performed in R v4.0.5 following R scripts by Martin et al. (2008), and adapting some of them into SAS v9.4. First, each individual cardenolide was transformed to a Gaussian distribution using PROC TRANSREG in SAS v9.4 (computed best lambda from –4 to 4 by 0.01 intervals), and then all the data were standardized by the mean. Then we performed a MANOVA including the transformed and standardized concentration of all 28 individual cardenolides as response variables, with population as a fixed factor and family within population as a random factor. Mean squares of the covariance matrices between populations (MS_b) and families within population (MS_w) were estimated by dividing their corresponding sums of squares matrices retrieved from the MANOVA (SS_b and SS_w, respectively) each by the degrees of freedom at each hierarchical level. Then, the original null hypothesis of the Q_{ST}–F_{ST} was tested on whether the proportionality observed coefficient ρ_{MS} between MS_b and MS_w covariance matrices departs from its expected value under neutrality (ρ_{ST}) (Martin et al., 2008). Multivariate neutral ρ_{ST} and its confidence intervals were retrieved from F_{ST} estimated through bootstrapping over loci as above (see main text, "Population differentiation and Q_{ST}–F_{ST} comparisons"). Neutrality is rejected if the 95% confidence intervals of ρ_{MS} are not overlapping those of ρ_{ST}. Confidence intervals for ρ_{MS} are calculated using the R scripts (Martin et al., 2008) through the maximum likelihood method.

Results

Phenotypic variation in cardenolide expression

Cardenolide expression varied strongly between tissues, populations, and genetic families (Figures 1 and 2, Supplementary Table S2) when plants were grown in a common environment. Total cardenolide concentration and cardenolide

richness both increased nearly sixfold from roots and leaves to seeds and were 20% higher in EH compared to DBP (Figure 1, Supplementary Table S2). In contrast, the polarity index increased 30% from seeds to roots and in a similar manner for both populations (Figure 1, Supplementary Table S2). Tissue-specific differences between populations were found for total cardenolides in the seeds and for cardenolide richness and polarity in the roots (Figure 1). Among the 28 cardenolides found, half were expressed only in a single tissue (3 in leaves, 11 in seeds) (Supplementary Table S2). Overall, specific cardenolides showed large differences between tissues and between genetic families within populations, but little variation was found between populations (Supplementary Table S2). Multivariate expression of the 28 cardenolides showed substantial differences between tissues, especially between roots and leaves compared to seeds, the latter showing the greatest differences between populations (Figure 2, Supplementary Table S3).

Genetic correlations and genetic integration of cardenolides

We next investigated the extent to which phenotypic differences in cardenolide concentrations are driven by how the individual compounds are genetically correlated (using family BLUPs) within and between tissues for each population. Genetic correlations between tissues for individual cardenolides were abundant (26 of 36), especially more common in the EH population (15 of 19, 79%) than in DBP (11 of 17, 65%), and stronger for polar cardenolides (Figure 3, Supplementary Tables S4 and S5). Similar results were found for both Pearson (Figure 3, Supplementary Table S4) and Spearman (Supplementary Table S4) genetic correlations for the most polar cardenolides (i.e., lower retention times), but were more dissimilar for nonpolar cardenolides (i.e., higher retention times). Whereas negative relationships between cardenolide concentrations in roots and seeds were the most abundant and similar between populations (Figure 3, Supplementary Tables S4 and S5), the strength and direction of correlations of other cardenolides were more population specific. Significant genetic correlations between cardenolides within tissues were moderately abundant (Figure 3 and Supplementary Figure S1, 295 of 496, 59%). Although less represented cardenolides tend to show fewer significant correlations due to lower sample sizes, >80% of the estimated

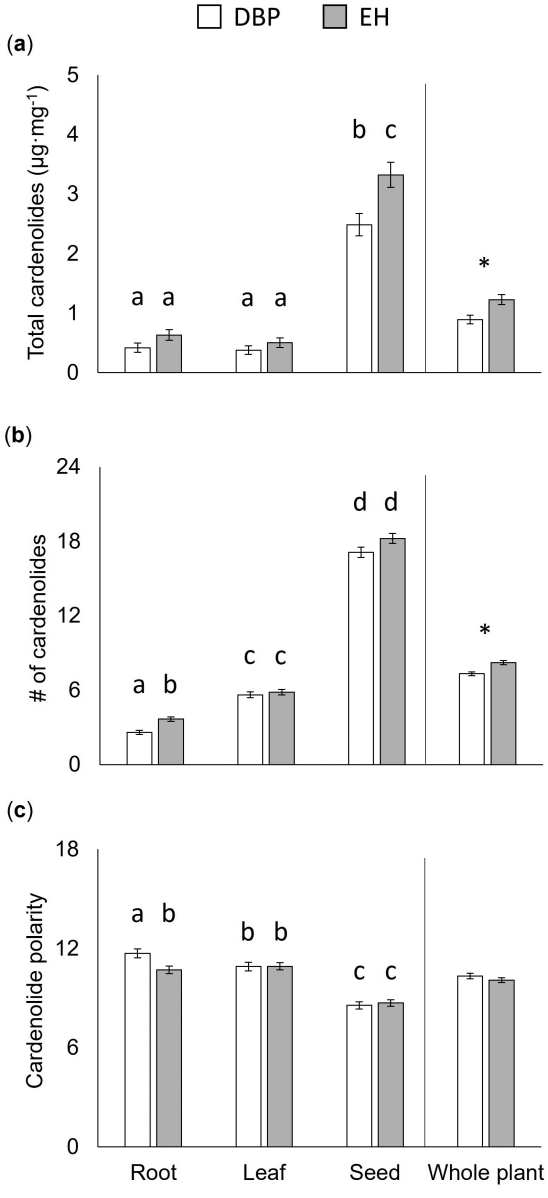

Figure 1. Comparison of the cardenolide chemistry across plant tissues between Durland Bird Preserve (DBP) and Ellis Hollow (EH) populations of common milkweed (*Asclepias syriaca*). Root and leaf tissue were taken from plants grown in a common environment. Summarized are total cardenolide concentrations (A), number of cardenolide peaks (B), and polarity index (C). Different letters and asterisks indicate significant differences between bars across tissues and at whole plant level, respectively ($p < .05$, Fisher's LSD). Bars and error bars represent back-transformed least square means ± *SE* (N = 65–74 per tissue per population) from mixed models.

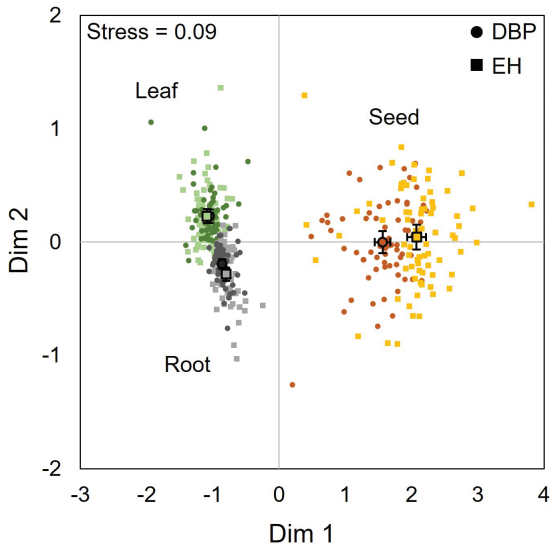

Figure 2. Non-metric multidimensional scaling (nMDS) of 28 cardenolides across plant tissues from individuals of Durland Bird Preserve (DBP, circles) and Ellis Hollow (EH, squares) populations of common milkweed (*Asclepias syriaca*). Dimension 1 shows greatest differences in seeds (dark and light orange) compared with root (dark and light gray) and leaf (dark and light green) chemistry, and also between population means (large, black-bordered symbols). Dimension 2 mostly represents differences in cardenolide composition between the three tissues. Error bars represent *SE*; N = 65-74 individuals for each population and plant tissue.

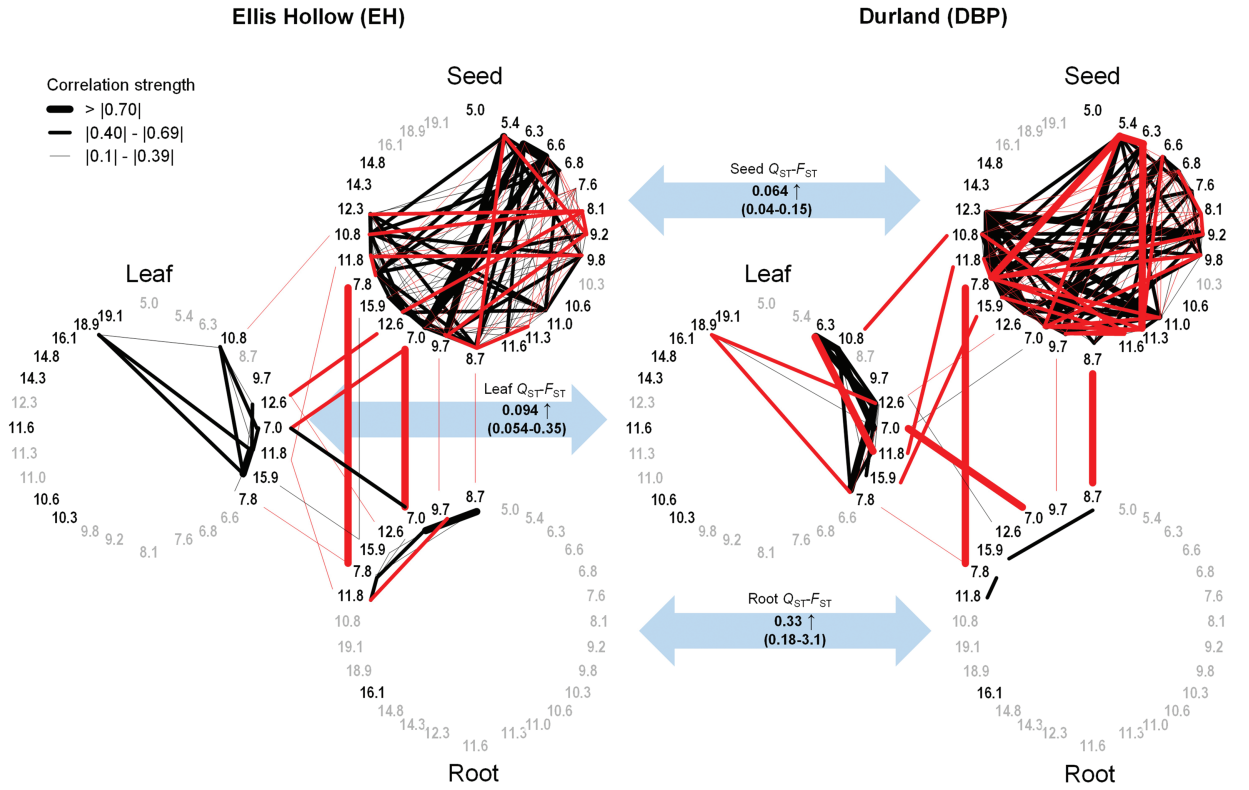

Figure 3. Genetic (full-sib) correlations between cardenolides within seed, leaf, and root tissues (within wheels), between tissues for each cardenolide within populations (between wheels), and tissue-specific divergent selection (↑ Q_{ST}–F_{ST}) in multivariate cardenolide chemistry (blue arrows) between the two populations (EH and DBP). Black numbers in wheels represent the retention times of individual cardenolides found in each tissue, whereas gray numbers were cardenolides not detected in that tissue. Black and red lines indicate positive and negative significant correlations (after correction using false discovery rate), respectively, and line thickness represents the strength of the correlation. N = 4–15 per correlation. Numbers in parentheses embedded in the blue arrows represent 95% confidence intervals of the Q_{ST}–F_{ST} estimate. All relationships represented are Pearson correlations (see also Supplementary Table S4 for correlations between tissues). For comparison with Spearman correlations between tissues, see Supplementary Table S5.

correlations (414) had between 10 and 15 families with nonzero values and the proportion of FDR-significant correlations (259, 63%) were similar between those sample sizes (Supplementary Figure S1).

Overall, the proportion of significant correlations were more prevalent in the seeds (63%) than in leaves (59.3%) and roots (25.9%) (Figure 3). This pattern was reflected in the genetic integration index for each tissue (Supplementary Figure S2), a measure of how strongly correlated the traits are in a multivariate sense. Cardenolide integration was 9- and 14-fold greater in seeds compared with roots and leaves, respectively, and was highest at the whole plant level (Supplementary Figure S2). There were no differences, nonetheless, in the degree of genetic integration between populations for any given tissue or at the whole plant level (Supplementary Figure S2). Since the genetic integration index does not provide information about the similarity (or dissimilarity) between correlation matrices of cardenolides, we compared correlation matrices between pairs of tissues within each population and between populations for each tissue by using a Monte Carlo procedure with 10,000 permutations. Among the nine possible pairwise matrix comparisons (i.e., root–leaf, root–seed, and leaf–seed within each population; EH-DBP for each tissue), we found neither evidence for the similarity between tissues within populations nor between populations for each tissue, except for the correlation structure of seed cardenolides between the two populations ($r_{observed}$ = 0.48 vs. $r_{permuted}$ = 0.04, p < .001).

Q_{ST}–F_{ST} comparisons

To test whether population differences in cardenolide chemistry were shaped by differential selection, we used univariate and multivariate Q_{ST}–F_{ST} approaches. First, neutral genetic variation, F_{ST}, was significant between the populations (F_{ST} = 0.013 [CI: 0.0088–0.0173], p < .001), and this was further confirmed by the hierarchical structure AMOVA of 971 SNPs (p < .001). Both multivariate and univariate comparisons showed that divergent selection (Q_{ST} > F_{ST}) is likely acting on the defensive cardenolome in a tissue-specific manner (Figure 3, Supplementary Table S6). Multivariate Q_{ST}–F_{ST} analyses showed signatures of divergent selection for all plant tissues (Figure 3) and at the whole plant level (Q_{ST}–F_{ST} = 0.37, CI: 0.24–0.82), but the magnitude was greater in the roots and entire plant, as indicated by their strong departure from neutrality. This pattern was similar to univariate Q_{ST}–F_{ST} analyses for composite measures of cardenolides—total concentration, cardenolide richness, and polarity index—in which divergent selection was detected for all variables in root tissue (Supplementary Table S6).

Signatures of population divergence were found for 11 of 28 individual cardenolides, and departures from neutrality were tissue and cardenolide specific (Supplementary Table S6). Four cardenolides showed divergent selection in their expression in more than one tissue (1 in roots and leaves, and 3 in seeds and whole plant, Supplementary Table S6), whereas tissue-specific signatures of divergent selection in cardenolide

expression were detected for 7 compounds (1 of 10 toxins in the roots, 1 of 16 in the leaves, 3 of 24 cardenolides in the seeds, and 1 of 28 at the whole plant level, Supplementary Table S6). Two cardenolides stood out in their patterns of population differentiation: Cardenolide 7.0 (glycosylated aspecioside, a polar cardenolide) showed a large departure from neutrality only in the roots, whereas 15.9, a thiazoline ring containing cardenolide (labriformin, the most nonpolar cardenolide in *A. syriaca*), showed a pattern of strong divergent selection only at the whole plant level (Supplementary Table S6).

Discussion

The asymmetric nature of interactions between individual host plant species and multiple herbivores suggests that the evolution of defense is most commonly diffuse in nature (Maddox & Root, 1990; Simms, 1990; Strauss et al., 2005; Wise & Rausher, 2013). In such scenarios, the direction and magnitude of selection by a given herbivore will depend on the selection by other attackers as well. This complexity represents an evolutionary challenge for the plant, especially because virtually all tissues can be attacked by herbivores. Nonetheless, if plants can compartmentalize defenses against their herbivores, then it may be possible that independent, tissue-specific defenses can evolve (Lopez-Goldar et al., 2022). At larger scales, interpopulation differences in selection may shape how defensive traits are correlated as well, favoring local adaptation (Lowry et al., 2019; Prasad et al., 2012). Here we show that genetic correlations between specific cardenolides differ among common milkweed tissues, being scarce in roots, moderate in leaves, and abundant in the seeds. Although the concentration of individual cardenolides was moderately correlated between tissues, the strength and direction of these correlations were population specific. These differences in prevalence, direction, and strength of genetic correlations likely resulted from responses to divergent selective pressures between populations through the evolution of the cardenolome in a tissue- and toxin-specific manner.

Phenotypic variation in the cardenolome is reflected in their underlying tissue-specific genetic correlation structure

Previous work has reported large differences in defense allocation between plant parts (Lopez-Goldar et al., 2022; McCall & Fordyce, 2010; Zangerl & Rutledge, 1996), with greater investment in tissues more closely tied to fitness (e.g., fruits, seeds) and those subject to higher probability of attack (Maynard et al., 2020; Zangerl & Rutledge, 1996). Our results point towards this notion, in which seeds showed the greatest cardenolide concentrations compared to roots and leaves in both populations. Previous work showed that greater allocation of defenses in seeds compared to other tissues could be explained by greater selective pressure exerted by highly cardenolide-tolerant herbivores, such as the seed bug, *Oncopeltus fasciatus* (Lopez-Goldar et al., 2022). Interestingly, the two populations also differed in both total cardenolide concentrations and multivariate chemistry only in the seeds, suggesting differences in herbivore pressure between locations. Alternatively, maternal effects may have contributed to phenotypic differences in plant defense, especially in seeds. Greater levels of defense are often detected in seedlings from damaged maternal plants, but these generally decrease over time as the plant develops when herbivory cues are absent from the environment (Agrawal, 2002; Sobral et al., 2021). Since leaves and roots were sampled from plants grown in a controlled environment with no damage imposed, and we were careful to avoid pest activity eliciting plant responses, we can reasonably assume that maternal effects had a minor contribution to defense compounds; nonetheless, cardenolide concentrations in seeds could have been affected by natural conditions since they were collected from plants in the field. Although we recommend caution for interpreting the seed results, heterogeneous allocation of defenses along the plant may occur due to distinct patterns of defense expression between tissues as a result of differential selection.

Plant resistance to herbivory can evolve thanks to the concerted expression of defense traits shaped by selection (Damian et al., 2020; Simms, 1990). Therefore, how suites of traits are genetically correlated may help us understand how plant defenses are responding to selection by their herbivore community. Whereas positive genetic correlations between plant defenses may indicate similar impacts of distinct defenses on a diverse array of enemies (Krischik et al., 1991), negative correlations between defenses may likely point toward plant evolutionary trade-offs in defending against multiple attackers (Simms & Rausher, 1989; Strauss et al., 2002). Nonetheless, since plant tissues are often attacked by diverse herbivores, tuning tissue-specific defenses may be beneficial if possible (Zangerl & Rutledge, 1996).

Our results showed contrasting differences in the genetic correlation structure of cardenolides between tissues. On the one hand, genetic correlations were scarce within roots and moderately abundant in leaves, suggesting that the evolution of individual cardenolides may not be strongly constrained (Gould, 1988; Strauss et al., 2005). For instance, milkweed leaves are eaten by insects in at least four different taxonomic orders that also vary dramatically in their adaptive mechanisms to circumvent plant defenses (Agrawal et al., 2012b; Taverner et al., 2019). Recent work suggests that, despite their known negative impact against herbivores overall, independent evolution of specific cardenolides may be proceeding due to differences in their effectiveness against distinct herbivores varying in tolerance strategies (Agrawal & Hastings, 2023; Agrawal et al., 2022; Lopez-Goldar et al., 2022). On the other hand, seeds showed more abundant and positive genetic correlations among cardenolides compared with roots and leaves, particularly between more polar compounds. Apart from possible maternal-related effects (see above), this result may suggest that those compounds are being favored (or subject to correlated selection) for greater concentrations in the seeds in response to herbivory.

It is possible that the patterns we observed for the seeds were mediated by plant physiological constraints in the expression or mobilization of toxins. Biosynthesis and mobilization of defenses may be restricted to more water-soluble compounds (i.e., polar) through the vascular system, whereas more nonpolar defenses may require alternative secretory structures (e.g., resin ducts, laticifers) (Gershenzon & Ullah, 2022). Nonpolar cardenolides seem to be restricted to mobilization through latex canals (Seiber et al., 1983), which are not expressed in seeds. Our findings support this idea, as the polarity index of seed cardenolides was the lowest of all tissues (i.e., greater proportion of polar cardenolides). Additionally, our finding of negative genetic correlations between seeds and other tissues only for more polar

cardenolides, especially in EH population, is consistent with the notion that biosynthesis and transport of toxins to different tissues may be restricted. A possible explanation is that greater mobilization of more polar cardenolides to the seeds from other tissues is driven indirectly by physiological constraints in the allocation of nonpolar cardenolides to seeds. In the tropical milkweed (*Asclepias curassavica*), for example, overaccumulation of more polar (i.e., less toxic) cardenolides in the seeds in response to highly insensitive herbivores (like the large milkweed seed bug, *O. fasciatus*) may be due to the restricted ability to allocate nonpolar cardenolides to seed tissues (Lopez-Goldar et al., 2022).

Alternatively, active transport of secondary metabolites through the vascular system or tissue-specific production of toxins may alleviate constraints in defense expression between organs. For instance, glucosinolate biosynthesis and transport between tissues impacted caterpillar performance and preference for old leaves in *Arabidopsis* (Hunziker et al., 2021). In another study, *Arabidopsis* responded to aphid herbivory by producing de novo specific, aphid-deterrent glucosinolates in the phloem (Kim & Jander, 2007). In our case, since biosynthetic pathways of specific cardenolides are still poorly characterized, it is challenging to rule out whether their expression is due to local biosynthesis or transport from one tissue to another (Alani et al., 2021).

Evidence of toxin- and tissue-specific divergent selection and speculation for their ecological interpretation

Although patterns of plant local adaptation are pervasive in nature (López-Goldar & Agrawal, 2021; Moreira et al., 2018; Schemske et al., 2009), most tests of local adaptation on traits that are expressed in multiple plant parts (e.g., plant toxins) were performed with single tissues. Since we show that toxin expression is highly tissue independent, we hypothesized that patterns of selection of cardenolide chemistry among populations may differ between tissues. Indeed, we found evidence of tissue-specific selection between the two milkweed populations, likely acting on the cardenolome. The magnitude of divergence increased from seeds to leaves to roots. This result contrasts with the patterns found for the genetic integration across tissues, which increased from roots to seeds. We speculate that these opposing trends between signatures of selection and genetic integration across tissues are in line with the notion that seed cardenolides may be more evolutionarily or physiologically constrained (see above). In addition, the high similarity in the correlation structure of seed cardenolides between populations and the dissimilarity in those of roots and leaves support our differences in the magnitude of genetic divergence across tissues and highlight the potential of shared evolutionary constraints in seed chemistry between populations. Given that seeds showed the greatest phenotypic differences between populations, it is also possible that we are observing the evolutionary outcome of a tissue that has already undergone strong population differentiation (Futuyma & Moreno, 1988). Nonetheless, it is important to interpret these results with caution because of possible maternal effects on seed cardenolides (which has not been investigated to our knowledge). Maternal effects can have a strong influence on plant defense levels in the offspring (including seeds) to prevent future herbivory (see below Caveats, limitations and conclusions) (e.g., Agrawal, 2002; Holeski, 2007; Sobral et al., 2021). Nonetheless, both populations exist in open, regularly disturbed areas in which milkweeds are abundant and resprout naturally every year from belowground rhizomes. Substantial evidence from past work indicates within- and between-population genetic variation for common milkweed traits (Agrawal, 2005; Agrawal et al., 2015; Woods et al., 2012). Also, despite the proximity of the populations, our results suggest that differential selection between the populations is stronger than the homogenizing effects of gene flow, as multivariate phenotypic differences between populations were from 5- to 28-fold greater (depending on the tissue or whole plant level) than the expected differentiation under neutrality (F_{ST}). Therefore, it is likely that differences in herbivore pressures between the populations are the main driver of the tissue-specific genetic (and potentially to some extent epigenetic) divergence of cardenolide chemistry.

Toxin- and tissue-specific signatures of population divergence for individual cardenolides paralleled the multivariate cardenolome patterns. All composite cardenolide variables in the roots showed signatures of divergent selection with greater magnitude in comparison to leaves and seeds, supporting our multivariate findings that selection is likely stronger belowground. Furthermore, the variable signatures of population divergence of individual cardenolides suggest different responses to selection for each toxin. Recent work linked distinct adaptive clinal patterns of individual cardenolides from seeds of common milkweed populations under divergent selection to their functional evidence against herbivore specialists (Agrawal et al., 2022). This result supports the notion that different cardenolides can be under distinct selection regimes, potentially contingent on the herbivore's ability to overcome plant defenses (Agrawal et al., 2012b; Taverner et al., 2019). Among all cardenolides that showed deviations from neutrality in our study, two cardenolides stood out for their patterns and magnitude of divergence: glycosylated aspecioside (a very polar compound), showed divergent selection only in the roots, and labriformin (relatively nonpolar, and reported highly toxic against milkweed specialist herbivores) (Agrawal et al., 2022), showed divergent selection only at the whole plant level.

Although more polar cardenolides were reported to be less toxic against specialized milkweed herbivores overall (Agrawal et al., 2012b, 2021; Jones et al., 2019), differences in insect tolerance to more versus less toxic cardenolides are reduced in less specialized milkweed herbivores, like root beetles (*Tetraopes* spp.) (Dalla et al., 2013; Lopez-Goldar et al., 2022). Since glycosylated aspecioside is the dominant, polar compound in common milkweed seeds (51% of the total concentration), its associated biosynthetic costs are expected to be relatively low. Therefore, the plant may benefit from greater concentrations of this cardenolide in the roots in response to belowground herbivory, but not likely in other tissues where more cardenolide-tolerant insects feed (e.g., monarchs on the leaves, large milkweed bugs on the seeds; Agrawal et al., 2012b; Lopez-Goldar et al., 2022).

Previous work on labriformin reported a geographical cline of increasing concentrations of this toxin in the seeds, with divergent selection likely acting between populations (Agrawal et al., 2022). Nonetheless, here we found a strong signature of divergent selection at the whole plant level, indicating that overall labriformin concentration in the plant is under heterogeneous selection between populations. This suggests that the expression of labriformin is likely not constrained to a specific tissue and that evolutionary patterns of

plant sub-individual variation in the allocation of defenses may change depending on the scale. Indeed, labriformin appears to be evolutionary relevant in milkweed–herbivore interactions (Agrawal & Hastings, 2023; Agrawal et al., 2022; Brower et al., 1982). Although we do not demonstrate the selective drivers behind population differentiation, we speculate that toxin- and tissue-specific evolutionary patterns of cardenolides may likely be resulting from heterogeneous pressures by distinct herbivores, favoring local adaptation of populations even at small spatial scales.

Limitations and conclusion

Recent evidence found strong divergence in phenotypic traits between two populations of wild and domesticated teosinte (1 km apart), both in the univariate and multivariate sense (Yang et al., 2019). Despite finding signatures of divergent selection using a similar sampling design in terms of number of populations, and our analytical efforts in providing robustness to our inferences, our study is more limited in terms of familial replication within the populations. Nevertheless, previous work in this and other milkweed species that used more genetic families also found that responses to selection for defense traits against distinct herbivores seem to be possible (Agrawal & Van Zandt, 2003; Agrawal et al., 2008; Bingham & Agrawal, 2010). Hence, and despite using more conservative approaches given our limitations (e.g., multivariate Q_{ST}–F_{ST} following Martin et al., 2008), there appears to be potential for fine-grained patterns of evolution in defense expression among plant tissues of common milkweed. Such differentiation may be common in nature and could likely be exacerbated in coevolved systems, given both the asymmetry and diversity of adaptive mechanisms among the interacting species.

Supplementary material

Supplementary material is available online at *Evolution*.

Data availability

SNP database, raw cardenolide data and the SAS code are publicly available at Zenodo repository (doi: 10.5281/zenodo.8219067).

Author contributions

X.L.G. and A.A.A. conceived and designed the research. X.L.G. performed the experiment, HPLC-UV chemical analysis, statistical analyses of cardenolide and SNP data, and wrote the manuscript with input from A.A.A. All authors approved the final version for publication.

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Conflict of interest

The authors declare no conflict of interest.

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