Macroevolution of leaf defenses and secondary metabolites across the genus Helianthus

Chase M. Mason1, Alan W. Bowsher1, Breanna L. Crowell1, Rhodesia M. Celoy2, Chung-Jui Tsai2 and Lisa A. Donovan1

1Department of Plant Biology, University of Georgia, Athens, GA 30602, USA; 2Warnell School of Forestry and Natural Resources, and Department of Genetics, University of Georgia, Athens, GA 30602, USA

Summary

• Leaf defenses are widely recognized as key adaptations and drivers of plant evolution. Across environmentally diverse habitats, the macroevolution of leaf defenses can be predicted by the univariate trade-off model, which predicts that defenses are functionally redundant and thus trade off, and the resource availability hypothesis, which predicts that defense investment is determined by inherent growth rate and that higher defense will evolve in lower resource environments.
• Here, we examined the evolution of leaf physical and chemical defenses and secondary metabolites in relation to environmental characteristics and leaf economic strategy across 28 species of Helianthus (the sunflowers).
• Using a phylogenetic comparative approach, we found few evolutionary trade-offs among defenses and no evidence for defense syndromes. We also found that leaf defenses are strongly related to leaf economic strategy, with higher defense in more resource-conservative species, although there is little support for the evolution of higher defense in low-resource habitats.
• A wide variety of physical and chemical defenses predict resistance to different insect herbivores, fungal pathogens, and a parasitic plant, suggesting that most sunflower defenses are not redundant in function and that wild Helianthus represents a rich source of variation for the improvement of crop sunflower.

Introduction

Plants and their herbivores constitute over half of all described species, and the evolutionary arms race between these two groups is hypothesized to explain much of the enormous global diversity in plant and herbivore species (Futuyma & Agrawal, 2009). Key plant traits known as defenses work to reduce herbivore damage and improve fitness in the face of herbivore pressure (Stamp, 2003; Boege & Marquis, 2005; Carmona et al., 2011). The evolution of such defenses is known to have resulted in adaptive radiations (Farrell et al., 1991; Agrawal et al., 2009a), and plant–herbivore interactions have become a major focus of evolutionary ecology (Agrawal, 2011; Johnson, 2011). Because plants mediate the primary flow of energy into almost all terrestrial ecosystems, the plant–herbivore interaction is the foundation of nearly all terrestrial food webs and is thus arguably one of the most important ecological processes on Earth. In addition to reducing herbivory, many plant defenses also reduce susceptibility to pathogen attack (Levin, 1976; Lattanzio et al., 2006; Treutter, 2006), and thus play multiple roles in determining plant fitness under pressure from natural enemies. Consequently, both physical and chemical defenses have been demonstrated to mediate adaptive differentiation among plant populations and species (Fine et al., 2004; Agrawal et al., 2012; Züst et al., 2012). Over the past half century, dozens of explanations have been put forward to account for the broad interspecific variation observed in plant defenses, although few have withstood empirical assessment across multiple systems (Stamp, 2003; Agrawal, 2007; Johnson, 2011). When considering the evolutionary radiation of a group of species into broadly distributed and environmentally variable habitats, two major explanations of defense variation make the most testable predictions: the univariate trade-off model and the resource availability hypothesis.

The univariate trade-off model is central to many major defense hypotheses (Agrawal, 2007). Because investment in defense comes at the expense of growth (Bazzaz et al., 1987), the univariate trade-off model predicts that trade-offs will exist among defenses such that only one defense performing a particular protective function will be invested in by a plant at any given time, and all comparable redundant defenses will not be allocated resources because of energetic cost (Agrawal, 2007). Many examinations of species at community and global scales have explored the possibility of trade-offs occurring among various defenses, but have generally found mixed support for univariate trade-offs
By contrast, if effective plant defense is conferred by multiple defense traits acting together, we should expect positive covariation among certain subsets of traits, forming defense syndromes in which distantly related species adopt similar multivariate defense phenotypes (Agrawal & Fishbein, 2006; Agrawal, 2007). Potential examples of such syndromes include convergent strategies for defending against different feeding guilds of herbivores — such as chewing versus piercing-sucking insects — or convergent defense strategies favored in different habitats or in conjunction with different life history characteristics. The largest world-wide analysis to date of univariate trade-offs and defense syndromes in a phylogenetic context found little evidence for either trade-offs or syndromes at a global scale, but hypothesized that both could exist at lower levels of evolutionary organization, such as within individual genera (Moles et al., 2013).

The resource availability hypothesis (also known as the growth rate hypothesis) attempts to explain variation in plant defense through two primary mechanisms — inherent growth rate and resource availability. First, this hypothesis predicts that defense variation occurs as a result of differences in the cost–benefit ratio of investment in defenses as a function of inherent plant growth rate (Coley et al., 1985; Coley, 1987; Stamp, 2003; Endara & Coley, 2011). For inherently fast-growing species, investing in defenses has the potential to reduce growth much more than in inherently slow-growing species, and fast-growing species can more quickly replace leaf area lost to herbivory or disease (Coley et al., 1985). Second, under this hypothesis the inherent growth rate of a species is assumed to be determined primarily by resource availability in the habitat to which it is adapted (Endara & Coley, 2011). Under this assumption, a plant species adapted to a resource-rich environment would benefit more from investing in growth than defense, as better access to water or nutrients renders herbivory less costly (Horsfall & Cowling, 1980). Conversely, a species adapted to a resource-poor environment would benefit more from a large investment in defenses, as resource limitation makes lost leaf tissue more difficult to replace. The resource availability hypothesis has been well supported by meta-analysis across studies of interspecific variation in defense (Endara & Coley, 2011), although there have been relatively few studies of this hypothesis in a phylogenetic context.

Because of the explicit linkage of defense investment with growth rate and environmental resource availability, the resource availability hypothesis also offers the potential for explaining leaf defense variation in relation to other aspects of leaf physiology. One major axis of variation in nondefense leaf physiology is the leaf economics spectrum, which describes suites of leaf traits governing carbon and nutrient investment and return that form ecological strategies ranging from resource-acquisitive to resource-conservative (Reich et al., 1997; Wright et al., 2004). This spectrum strongly predicts whole-plant growth rate and tolerance of low-resource conditions (Poorter et al., 1990; Sterck et al., 2006; Hallik et al., 2009; Nardini et al., 2012), such that the leaf economics spectrum should predict the interspecific differences in inherent growth rate that define differences in defense investment under the resource availability hypothesis. One would therefore expect species with a more resource-acquisitive leaf economic strategy to invest less in defense than species with a more resource-conservative strategy. However, the hypothesis of a leaf-level relationship between leaf economics and leaf defense investment has yet to be rigorously tested.

Here, we investigated the evolution of leaf defenses and putatively defensive secondary metabolites across the genus Helianthus (the sunflowers). Sunflowers are a diverse group of species that have radiated into a wide variety of habitats throughout North America, including deserts, grasslands and prairies, evergreen and deciduous forests, freshwater wetlands and salt marshes, coastal dunes, rock outcrops, and ruderal habitats (Heíser et al., 1969). Many studies have explored physiological adaptation to variation in abiotic environmental conditions in wild sunflowers (Rosenthal et al., 2002; Sambatti & Rice, 2007; Donovan et al., 2009; Kawakami et al., 2011; Brouillette et al., 2014; Mason & Donovan, 2015b), but little work has investigated how members of Helianthus have differentiated in response to biotic pressures such as herbivory or disease (Fig. 1). Here, we use a phylogenetic comparative approach to examine: (1) whether there are univariate trade-offs or syndromes in leaf defenses across Helianthus; (2) how leaf defenses relate to the leaf economics spectrum; (3) how leaf defenses vary across environmental gradients; and (4) how leaf defense traits predict resistance to known sunflower herbivores and pathogens, and accompanying plant performance under attack.

Materials and Methods

Study system

To examine the evolution of leaf defenses across Helianthus, we selected 28 diploid nonhybrid species to make use of the most

Fig. 1 Sunflower herbivory in the field. Leaf and floral damage by spotted cucumber beetle (Diabrotica undecimpunctata) on Helianthus petiolaris in Illinois, USA (a), and leaf damage by the eastern lubber grasshopper (Romalea guttata) on Helianthus agrestis in Florida (b) are shown.
recent and robust phylogeny of the diploid backbone of the genus (Stephens et al., 2015). These 28 species make up the vast majority of the diploid nonhybrids in the genus (34 total), and represent over half of the genus as a whole, which contains c. 50 species in total (Heiser et al., 1969; Timme et al., 2007). To capture intraspecific variation, two to four populations were identified from across the documented geographic range of each of the 28 species (83 populations in total). Seeds from these populations were either wild-collected or obtained from accessions maintained by the USDA National Genetic Resources Program (Supporting Information Notes S1).

Plant growth

Leaf defenses are known to be highly environmentally labile (Lieurance & Cipollini, 2013; Moore et al., 2014). In order to minimize environmentally induced variation, a common garden approach was used, with plants grown under controlled high-resource glasshouse conditions. Although this approach does not eliminate the possibility of maternal effects from field-sampled seeds, maternal effects nevertheless contribute to trait variation among species and populations in nature; therefore, the potential inclusion of maternal effects in our study should not impact conclusions related to trait trade-offs and defense syndromes.

To accommodate all 83 populations, the 28 species were divided into two common garden experiments performed in the summers of 2012 and 2013 at the University of Georgia Plant Biology glasshouses. To minimize differences between the two common gardens, experimental timing was synchronized between years and environmental conditions were kept as similar as possible, including the use of identical pots, soil mixture, irrigation, fertilization, and glasshouse temperature controls. Three species representing a cross-section of life history and overall morphology (Helianthus annuus L., Helianthus radula (Pursh) Torr. & A. Gray, and Helianthus silphioides Nutt.) were grown in both years as phytometers to assess the seven leaf defense traits (Mason & Donovan, 2015a). Thus, each species was considered separately, and all populations and replicates of each species were sampled on a single day, once all replicate plants had produced at least four fully expanded leaf pairs but before the onset of reproductive activity (Mason & Donovan, 2015b). A single leaf was harvested from each replicate plant to assess the seven leaf defense traits described above, using established protocols as described in Methods S1.

Leaf defense measurements

Seven putative leaf defense traits were included in this study: thickness, toughness, dry matter content, trichome density, tannin activity, lipid content, and ash content. Thickness, toughness, and leaf dry matter content (LDMC) are common measures of leaf palatability and digestibility, with higher dry matter content, toughness, and thickness often correlating with reduced herbivory and herbivore growth (Scriber, 1977; Elger & Willby, 2003; Hanley et al., 2007; Kitajima & Poorter, 2010; Kitajima et al., 2012), although extremely low dry matter content (i.e. succulence) can also deter herbivory (Pérez-Harguindeguy et al., 2003; Moles et al., 2013). Trichomes block or ensnare small herbivores such as insects or gastropods, interfering with feeding or oviposition, and may keep pathogen-containing water droplets away from the leaf surface (Levin, 1973; Horsfall & Cowling, 1980; Hanley et al., 2007). Glandular trichomes are also known to secrete secondary compounds, which may deter small herbivores and inhibit the colonization and growth of pathogens (Levin, 1973; Horsfall & Cowling, 1980; Hanley et al., 2007). Tannins are polyphenolic compounds traditionally recognized for their protein precipitation capacity, and more recently for their strong oxidative activity against herbivores (Salminen & Karonen, 2011). Tannins are widespread in plants, and have been demonstrated to reduce herbivory by reducing leaf protein digestibility, damaging the digestive system, and generally interfering with metabolism and growth (Shimada, 2006; Roslin & Salminen, 2008; Spalinger et al., 2010; Moles et al., 2011). Tannins have also been implicated in pathogen resistance (Levin, 1976; Lattanzio et al., 2006). Leaf lipid content is a combined proxy for leaf oils, resins, and waxes (Moles et al., 2011), all of which deter herbivory and pathogen attack (Levin, 1976; Horsfall & Cowling, 1980; Coley et al., 1985; Lincoln, 1985; Markó et al., 2008). Leaf ash content reflects the relative presence of inorganic substances, including two major classes of compounds: calcium oxalates and silicate phytoliths (Lanning & Eleuterius, 1985; Franceschi & Nakata, 2005; Moles et al., 2011). These increase the abrasiveness of leaves, grinding down the mouthparts of chewing herbivores and disrupting their digestion, resulting in reduced growth rates (Korth et al., 2006; Massey et al., 2006; Hanley et al., 2007; Moles et al., 2011). Silicate phytoliths have also been demonstrated to increase resistance to fungal infection (Horsfall & Cowling, 1980; Cooke & Leishman, 2011). Together, these seven leaf traits reflect a wide variety of different classes and mechanisms of putative leaf defenses present in plants globally (Moles et al., 2013).

Leaf sampling was standardized by ontogenetic stage, in order to account for large variation among species in growth form, growth rate, and whole-plant development (Mason et al., 2013; Mason & Donovan, 2015a). Thus, each species was considered separately, and all populations and replicates of each species were sampled on a single day, once all replicate plants had produced at least four fully expanded leaf pairs but before the onset of reproduction (Mason & Donovan, 2015b). A single leaf was harvested from each replicate plant to assess the seven leaf defense traits described above, using established protocols as described in Methods S1.

In addition to defenses, two other previously reported leaf traits (Mason & Donovan, 2015b) are included here as descriptors of nondefense leaf physiology. These traits are carbon (C): nitrogen (N) ratio, a common measure of leaf palatability/
nutritional quality (Pérez-Harguindeguy et al., 2003; Agrawal & Fishbein, 2006), and leaf economic strategy, defined as the first principal component (LES PC1) capturing 51.5% of variation across the 28 *Helianthus* species in six leaf economic traits: photosynthetic and respiration rates, N and phosphorus concentrations, leaf mass per area, and leaf lifespan (Mason & Donovan, 2015b). These traits were measured simultaneously with the leaf defenses described above on the same replicate plants, and with the exception of leaf lifespan were all assessed on either the exact same leaf used for assessment of defense traits or the opposite leaf of the same leaf pair. C : N ratio and leaf economic strategy are therefore physiologically paired to the defenses of interest.

For each leaf trait, population least-squares means were calculated by ANOVA in order to account for the randomized complete block design. To check for any consistent differences between the two common gardens, trait values obtained for all replicates of the three species (nine populations) grown in both years as phytometers were assessed by ANOVA. ANOVAs run on individual replicate plants of each phytometer species (including year, population, and block effects with interactions) determined that no traits showed significant differences between years across all three phytometer species, as well as only one significant population × year interaction (for leaf lipid content in *H. annuus*). Accordingly, no corrections of traits between years were performed and least-squares trait means for the nine phytometer populations were averaged between the 2 years for use in subsequent analyses.

Secondary metabolite screening

To characterize broad secondary metabolite variation across species, we used a nontargeted profiling approach to capture variation in as many compounds as possible, rather than *a priori* selection of specific compounds or classes of interest. Previous studies of both wild and cultivated sunflowers have mostly focused on individual groups of charismatic compounds such as coumarins, flavonoids, or sesquiterpene lactones (Schilling & Mabry, 1981; Schilling, 1983; Rogers et al., 1987; Spring & Schilling, 1989; Olson & Roseland, 1991; Roseland & Grosz, 1997; Rowe et al., 2012), but to date no study to our knowledge has characterized broad variation in secondary metabolites across the wild sunflowers.

In brief, a single leaf was collected from each plant, snap-frozen in liquid nitrogen, and stored at −80°C. Samples were ground in liquid nitrogen, and 10 mg of freeze-dried leaf powder was extracted in 400 µL of 1:1 methanol : chloroform (v/v) containing three internal standards (13C6-cinnamic acid, D2-benzoic acid, and resorcinol) by sonication in ice-chilled water for 30 min. After the addition of 200 µL of high-performance liquid chromatography (HPLC)-grade water, samples were vortexed and centrifuged, and the aqueous layer was collected and re-centrifuged. Leaf extracts were analyzed by reverse-phase high-performance liquid chromatography–mass spectrometry (HPLC-MS) as described in Xue et al. (2013) and in Methods S1. Individual samples from four replicate plants per population were initially included for HPLC-MS analysis, although this was reduced to an average of 3.77 because of instrument breakdowns.

In total, HPLC-MS analysis yielded 1744 distinct peaks across all species, hereafter referred to as the HeliaMet database (Notes S2). Populations were scored for the presence of each of these compounds, defined as presence in at least 75% of replicates in a population to limit false positives. Presence/absence data were used to determine the number of compounds present in each population, as well as to perform principal coordinates analyses at the population level, in order to describe overall variation in secondary metabolite composition as the first two axes of variation (HeliaMet PCO1 and HeliaMet PCO2). While these population-level metrics were used to investigate correlated evolution between secondary metabolite composition and leaf-level defenses and environmental characteristics, species-level metrics were needed in order to investigate evolutionary correlations with species-level estimates of resistance to herbivores, pathogens, and parasites. To obtain these, species were scored for the presence or absence of compounds, defined as presence in any population by the criteria used at the population level, and the average number of compounds present in each species was calculated. Principal coordinates analysis was repeated at the species level to calculate the first two axes of variation (HeliaMet PCO1 and HeliaMet PCO2).

To characterize the most common secondary metabolites across *Helianthus*, all compounds present in at least 20 species were selected for further analyses. For this subset of 22 compounds, mean peak areas for each compound were calculated for each population, and species means were calculated from population means. Normalized peak area data were used to perform principal components analyses at the population and species levels, in order to describe overall quantitative variation in the most common secondary metabolites as the first two axes of variation (HeliaMet Subset PC1 and HeliaMet Subset PC2). These two axes together explained 47.4% of quantitative variation among populations in the 22 focal compounds, and 54.9% of variation among species. Putative compound identities for the 22 focal compounds (Table S1) were determined by comparing with authentic standards where possible, by searching predicted m/z ratios in the METLIN database (Smith et al., 2005), or by comparison with predicted m/z ratios of compounds detected in previous studies of sunflower (Tal & Robeson, 1986; Spring & Schilling, 1989; Olson & Roseland, 1991; Rowe et al., 2012), with specific focus on coumarins and terpenoids (including sesquiterpene lactones).

Environmental data

Soil and climate characteristics were obtained for the source site of each population, as reported previously in Mason & Donovan (2015b), and detailed in Methods S1. In brief, five soil cores were collected at representative locations throughout each population to a depth of c. 20 cm, dried at 60°C and homogenized before analysis for soil fertility characteristics, with replicate cores averaged to generate site means for use in subsequent analyses.
Climate data were obtained for each population source site using the WorldClim database (Hijmans et al., 2005) and the CGIAR Global Aridity and PET database (Zomer et al., 2008).

Herbivore and pathogen resistance

Wild *Helianthus* species have long been screened for herbivore and pathogen resistance, primarily with the intention of identifying useful germplasm for improved breeding of crop sunflower. Here, we made use of data from seven such studies, yielding species-level data on responses to a variety of common sunflower pests and diseases. These include the aphid *Masonaphis masoni* (Rogers & Thompson, 1978), the western potato leafhopper *Empoasca abrupta* (Rogers, 1981), the painted lady butterfly caterpillar *Vanessa cardui* (E. W. Goolsby et al., unpublished), the parasitic broomrape plant *Orobanche cernua* (Ruso et al., 1996), leaf blight caused by *Alternaria helianthi* (Morris et al., 1983), head rot caused by *Rhizopus* species (Yang et al., 1980), and powdery mildew caused by *Erysiphe choricarcarum* (Salimen et al., 1982). In all these studies, a cross-section of species were challenged with the focal pest or disease under experimental field or glasshouse conditions, and responses recorded. Aggregated data from these studies can be found in Notes S1.

Phylogenetic and clustering analyses

All phylogenetic analyses were performed with the most recent phylogeny of the genus *Helianthus*, which was built using 170 nuclear genes and is well supported (Stephens et al., 2015). The tree topology was inferred by the coalescent method MP-EST (maximum pseudo-likelihood estimation of species trees), with branch lengths fitted to that topology using maximum likelihood in RAxML (Stephens et al., 2015).

To assess pairwise macroevolutionary correlations among traits and between traits and environmental characteristics, we used phylogenetic mixed models on population trait means to account for within-species trait variation (Felsenstein, 2008). Under this method, within- and among-species trait covariances are estimated iteratively using an expectation-maximization algorithm assuming a Brownian motion model of evolution (Felsenstein, 2008). Tests comparing Brownian motion models of evolution to a star-phylogeny model found mixed support among individuals (Revell, 2012). The *phylogenospace* function in *phytools* was used to visualize the evolution of the two primary axes of secondary metabolite variation across *Helianthus*. To assess trait differences between species, the *fastAnc* function in *phytools* was implemented on correlations in each set of analyses: trait–trait correlations, trait–environment correlations, and trait–resistance/performance correlations.

Maximum likelihood ancestral state reconstructions were performed with the *fastAnc* function in the R package *phytools* (Revell, 2012). The *phylogenospace* function in *phytools* was used to visualize the evolution of the two primary axes of secondary metabolite variation across *Helianthus*. To assess trait differences between species, the *fastAnc* function in *phytools* was performed with the *glm* function in the R package *nlme* (Pinheiro et al., 2014), with post hoc tests performed with the *glht* function in the *multcomp* package (Hothorn et al., 2008). To assess the presence of syndromes of defenses across species, we employed the general approach of Agrawal & Fishbein (2006). Both principal components analysis and standardized hierarchical clustering analysis (Ward method) were performed on species trait means in JMP PRO v.11 (SAS Institute, Cary, NC, USA). To test for significant clustering of species by defenses, the similarity profile routine (SIMPROF) was implemented using Euclidean distance with 9999 permutations in the software package PRIMER v.7 (Clarke et al., 2008; Moles et al., 2013).

Results

Correlated evolution of leaf defenses across *Helianthus*

Among leaf-level defenses, most of the significant macroevolutionary correlations between traits exhibit $R^2 < 0.25$ (Table 1), suggesting a general lack of direct mechanistic relationships between defense traits (Poorter et al., 2014). However, there are several important exceptions to this finding. First, leaf thickness is strongly negatively related to LDMC and strongly positively related to ash content, indicating repeated evolution of high leaf thickness in more succulent-leaved species and those with high ash content (Table 1). Second, ash content is positively correlated with leaf toughness, indicating that ash content probably contributes to leaf toughness across *Helianthus* (Table 1). Third, tannin activity is strongly positively correlated with leaf C:N ratio, indicating repeated evolution of high protein-precipitation capacity in leaves with low N reserves (Table 1). While there are strong relationships among some individual defenses, the low proportion of significant macroevolutionary correlations is not what would be expected in the presence of distinct defense syndromes. Additionally, hierarchical cluster analysis yielded no significant clustering of species (SIMPROF; $\pi = 2.55$; $P = 0.432$), and principal components analysis of defenses showed that multivariate trait space is continuously filled by species, rather than displaying groups of species as would be expected if defenses formed strong syndromes (Fig. 2).

In general, lower defense is associated with a more resource-acquisitive leaf economic strategy (as defined by LES PC1; Mason & Donovan, 2015b). The evolution of more resource-acquisitive leaves is correlated with lower toughness and higher LDMC, and strongly correlated with lower tannin activity.
Table 1 Macroevolutionary correlations among leaf physical and chemical defenses, carbon : nitrogen (C : N) ratio, leaf economic spectrum (LES) strategy, and HeliaMet metrics of secondary metabolite variation as assessed by phylogenetic mixed model

|                      | Trichome density | Thickness | Toughness | LDMC | Tannin activity | Lipid content | Ash content | C : N ratio | LES PC1 | HeliaMet PCO1 | HeliaMet PCO2 | Avg. no. of compounds | HeliaMet Subset PC1 | HeliaMet Subset PC2 |
|----------------------|------------------|----------|-----------|------|----------------|---------------|-------------|-------------|----------|-----------|-------------|----------------|---------------------|--------------------|--------------------|
| Thickness            | (+)0.12          |          |           |      |                |               |             |             |          |           |             |              |                     |                    |                    |
| Toughness            | (-)0.00          | (+)0.16  | (-)0.00   |     |                |               |             |             |          |           |             |              |                     |                    |                    |
| LDMC                 | (+)0.00          | (-)0.61  | (-)0.17   |     |                |               |             |             |          |           |             |              |                     |                    |                    |
| Tannin activity      | (-)0.01          | (-)0.02  | (+)0.05   | (-)0.03 |                |               |             |             |          |           |             |              |                     |                    |                    |
| Lipid content        | (+)0.00          | (-)0.19  | (+)0.00   | (+)0.13 | (-)0.03        |               |             |             |          |           |             |              |                     |                    |                    |
| Ash content          | (+)0.03          | (+)0.51  | (+)0.30   | (-)0.20 | (-)0.03        | (-)0.11       |             |             |          |           |             |              |                     |                    |                    |
| C : N ratio          | (-)0.00          | (+)0.01  | (+)0.21   | (-)0.13 | (+)0.77        | (-)0.01       | (-)0.01     |             |          |           |             |              |                     |                    |                    |
| LES PC1              | (-)0.00          | (-)0.07  | (-)0.20   | (+)0.20 | (-)0.50        | (+)0.06       | (-)0.77     |             |          |           |             |              |                     |                    |                    |
| HeliaMet PCO1        | (+)0.06          | (+)0.26  | (+)0.16   | (-)0.05 | (+)0.00        | (+)0.05       | (+)0.24     | (+)0.11     | (-)0.13 | (-)0.05 |             |              |                     |                    |                    |
| HeliaMet PCO2        | (+)0.02          | (+)0.00  | (-)0.07   | (+)0.05 | (-)0.22        | (+)0.06       | (+)0.03     | (-)0.18     | (+)0.26 | (-)0.08 |             |              |                     |                    |                    |
| Avg. no. of compounds| (-)0.01          | (-)0.17  | (-)0.12   | (+)0.05 | (-)0.02        | (+)0.17       | (-)0.44     | (-)0.12     | (+)0.11 | (-)0.82 | (+)0.08     |              |                     |                    |                    |
| HeliaMet Subset PC1  | (-)0.01          | (-)0.09  | (-)0.01   | (+)0.01 | (-)0.07        | (-)0.08       | (+)0.27     | (-)0.04     | (-)0.05 | (-)0.02 | (-)0.56     | (-)0.00     |                     |                    |                    |
| HeliaMet Subset PC2  | (-)0.00          | (-)0.20  | (-)0.02   | (+)0.51 | (-)0.15        | (+)0.08       | (-)0.25     | (+)0.44     | (-)0.42 | (+)0.08 | (+)0.33     | (-)0.02     |                     |                    |                    |

$R^2$ and directionality of correlations are presented, with those found to be significant at $P<0.05$ in blue, and those found to be also significant at the more stringent multiple comparisons cutoff (false discovery rate; Benjamini & Hochberg, 1995) in red.

LDMC, leaf dry matter content; PC1, PC2, first and second principal components axes; PCO1, PCO2, first and second principal coordinate axes.

Table 1. Macroevolutionary correlations among leaf physical and chemical defenses, carbon : nitrogen (C : N) ratio, leaf economic spectrum (LES) strategy, and HeliaMet metrics of secondary metabolite variation as assessed by phylogenetic mixed model.

As expected, leaf economic strategy is a strong predictor of leaf palatability as defined by the C : N ratio (Table 1). However, leaf economic strategy is not evolutionarily correlated with trichome density, leaf thickness, leaf ash content, or leaf lipid content (Table 1). In addition, no leaf-level defenses significantly differ among growth forms or life histories by PGLS-ANOVA.

Secondary metabolite variation with defense traits and life history

Among metrics of secondary metabolite composition, HeliaMet PCO1 correlates strongly negatively with the average number of compounds detected ($R^2 = 0.82$; Table 1), while HeliaMet PCO2 correlates strongly negatively with the abundance of the most common compounds as described by HeliaMet Subset PC1 ($R^2 = 0.56$; Table 1). HeliaMet PCO2 reflects a general differentiation between annual and perennial Helianthus species that is significant by PGLS-ANOVA (Fig. 3). With respect to leaf-level defenses, HeliaMet PCO1 correlates positively with thickness, toughness, ash content, and tannin activity, and C : N ratio, although none of these correlations are particularly strong ($R^2 ≤ 0.26$; Table 1).

The subset of 22 secondary compounds present in at least 20 species of Helianthus is dominated by phenylpropanoids, especially hydroxycinnamoyl-quinic acid conjugates (Table S1). The list also includes three putative coumarins (coumarin, scopolin, and scopoletin) as well as one putative terpenoid (ciliaran), all of which have been previously reported in sunflowers (Table S1; Tal & Robeson, 1986; Spring & Schilling, 1990; Olson & Roseland, 1991). The overall abundance of these 22 compounds, as described by HeliaMet Subset PC1 (Table S1, Fig. S1), correlates negatively with ash content but no other leaf-level defenses (Table 1). Conversely, HeliaMet Subset PC2 correlates strongly positively with LDMC and negatively with thickness, tannin activity, and C : N ratio (Table 1).

Environmental gradients in defense and secondary metabolites

Across Helianthus, there are many significant correlations between defenses and environmental characteristics. Shifts into warmer environments are associated with increased trichome density, leaf thickness, C : N ratio, and reduced lipid content, while only C : N ratio is associated with shifts in temperature seasonality (Fig. 4; Table S2). Shifts into wetter environments are associated with reduced toughness and ash content, and shifts into environments with more seasonal rainfall are associated with increased ash content (Fig. 4; Table S2). Lastly, shifts on to soils with higher organic matter, N, and phosphorus are associated with decreased thickness and ash content, as well as increased lipid content (Fig. 4; Table S2).

Secondary metabolite variation is also correlated with environmental characteristics. HeliaMet PCO1 (reflecting the average number of compounds) is associated with shifts in soil organic matter and N content, with higher average number of compounds detected in more fertile environments (Fig. 4; Table S2). Both HeliaMet PCO2 and HeliaMet Subset PC1 are associated with shifts in aridity index and precipitation seasonality, with higher abundance of the most common compounds in wetter and less seasonal environments (Fig. 4; Table S2). Additionally, HeliaMet Subset PC2 is correlated with mean annual temperature, temperature seasonality, and soil phosphorus (Fig. 4; Table S2).
Leaf defenses as predictors of herbivore and disease resistance

Many leaf-level defenses and metrics of secondary metabolite variation predict differential species responses to herbivores and disease reported in the literature. The proliferation of aphids (*Masonaphis masoni*) is negatively correlated with tannin activity, HeliaMet Subset PC1, and the average number of compounds (Fig. 5; Table S3). Among individual compounds, aphid abundance is negatively correlated with the abundance of several HeliaMet peaks putatively identified as hydroxycinnamoyl-quinic acid conjugates (Table S3). Contrary to expectations, trichome density, ash content, and C : N ratio are positively correlated with aphid proliferation (Table S3). Unlike aphids and leafhoppers, which both belong to the piercing/sucking feeding guild and are impacted strongly by chemical traits, herbivory by the chewing caterpillar larvae of the painted lady butterfly (*Vanessa cardui*) is strongly associated with physical defenses. The mass of leaf consumed in feeding trials is negatively correlated with both leaf toughness and ash content and strongly positively correlated with LDMC (Table S3). However, consumption by this herbivore also has the strongest correlations with individual secondary metabolites observed in this study, with p-coumaroylquinic acid and a compound putatively identified as syringic acid extremely predictive of consumption (Table S3).

With respect to disease resistance, leaf defenses and secondary metabolite variation do not strongly predict resistance to leaf blight by *Alternaria helianthii* or head rot by *Rhizopus* species (Table S4). However, resistance to powdery mildew (*Erysiphe*...
chicoracearum) is strongly predicted by the abundance of the most common secondary metabolites as described by HeliaMet Subset PC1 (Fig. 5; Table S4), and level of infection is strongly negatively correlated with the majority of phenylpropanoid derivatives (Table S4). Most leaf-level defense traits are not consistently correlated with powdery mildew disease index under both field and glasshouse conditions, although ash content is consistently positively correlated with the level of infection (Table S4). In a similar manner, resistance to the parasitic plant broomrape (Orobanche cernua) is not strongly predicted by most leaf-level defenses, with the exception of tannin activity, which is negatively correlated with the incidence of broomrape infection (Fig. 5; Table S4).

**Discussion**

Little evidence for trade-offs or syndromes in defense

Across *Helianthus*, there is little evidence for univariate trade-offs among leaf-level defense traits, as well as little evidence for recurring defense syndromes. In general, many different combinations of physical and chemical defenses have evolved across sunflowers. This supports the emerging model of plant defense that suggests that the overall function of defenses (to reduce total herbivore and pathogen pressure) is achieved by the totality of defenses present, and that investment in multiple defenses is unlikely to be redundant or wasteful (Agrawal, 2007, 2011). Rather than responding to individual defenses, herbivores and pathogens experience the full suite of defense traits in a multivariate way, allowing for individual defenses to evolve in a labile manner without compromising overall plant defense (Agrawal, 2007, 2011). This model of defense evolution explains why no strong global-scale trade-offs in defenses have been identified (Moles *et al.*, 2013), but also why recurring evolution of syndromes of defenses might be common within some genera but not others (Agrawal & Fishbein, 2006; Agrawal, 2011; Haak *et al.*, 2013; Johnson *et al.*, 2014).

Among the wild sunflowers assessed here, the most widespread group of secondary metabolites are the phenylpropanoids, with high levels evolving together repeatedly probably as a result of shared biosynthetic pathways (Salminen & Karonen, 2011). Some of these metabolites have been previously identified as the most abundant phenolics in crop sunflower seeds (Weisz *et al.*, 2009). Additionally, the three putative coumarins found to be widespread across the genus (coumarin, scopolin, and scopoletin) are known to contribute to herbivore and pathogen resistance in cultivated sunflower (Tal & Robeson, 1986; Olson & Roseland, 1991) and its wild progenitor (Roseland & Grosz, 1997). Our results suggest that phenolics and coumarins are abundant across the wild members of the genus as well, and thus probably play a role in herbivore and pathogen resistance as in crop sunflower. The relative lack of sesquiterpene lactones and other terpenoids may reflect limitations of the analytical procedures used in this study. Alternatively, the apparent limited distribution within the genus of individual sesquiterpene lactones and other terpenoids may reflect rapid diversification of these compounds within the genus, such that few individual compounds are shared across many species. Early work characterizing these compounds in wild
sunflowers found that many were detected in only a handful of species, and many more were variable within species (Spring & Schilling, 1989, 1990, 1991), suggesting that terpenoids indeed evolve more rapidly than phenolics and coumarins.

**Mixed support for the resource availability hypothesis across Helianthus**

Across *Helianthus*, species with more resource-conservative leaf economic traits supporting a slower-growth persistence strategy have tougher, more succulent leaves with higher tannin activity. Interestingly, these three defenses have previously been linked to shifts in leaf economic strategy through whole-plant ontogeny in three species of wild sunflowers (Mason & Donovan, 2015a). This suggests that relationships between leaf economic traits and these defenses hold across scales in *Helianthus*, and that investment in these defenses probably trades off with inherent growth rate. This fits with the first prediction of the resource availability hypothesis, with higher defense in species with leaf physiology supporting slower inherent growth (Endara & Coley, 2011). However, the second prediction of the hypothesis – that faster inherent growth rate is driven by higher resource availability – does not hold across *Helianthus* (Mason & Donovan, 2015b). For instance, while faster leaf economic strategy is generally positively related to metrics of soil fertility, faster leaf economic strategy is strongly negatively related to water availability (Mason & Donovan, 2015b). While we lack detailed information on which resources most limit growth across environments in *Helianthus*, it appears that water availability plays a relatively stronger role than soil fertility (Mason & Donovan, 2015b). In fact, some of the fastest growing annual sunflowers occur in arid deserts, while some of the slowest growing basal rosette perennials occur in the moist habitats of the southeastern USA (Mason & Donovan, 2015b). Given this, associations between defense traits and environmental characteristics were highly mixed in strength and direction and did not support the second prediction of the resource availability hypothesis. This pattern is likely to hold across habitats in many short-lived herbaceous plants, where drought-escape and other fast-growth strategies for overcoming abiotic stress often dominate low-resource habitats. This calls into question the applicability of the second prediction of the resource availability hypothesis for herbaceous plants, in contrast to the persistent woody species for which the hypothesis was originally developed (Coley *et al.*, 1985, Coley, 1987; Stamp, 2003).

Because many leaf defense traits also serve other physiological functions, environmental correlations should be interpreted with caution in relation to the resource availability hypothesis. Many defense traits may evolve primarily under selection from the abiotic environment, despite providing effective defense against herbivores (Agrawal, 2011). This may be the case across *Helianthus*, where for instance we find repeated evolution of higher trichome density with increasing mean annual temperature. Trichomes are known to be important for preventing water loss, excessive heat build-up, and the absorbance of harmful ultraviolet radiation, all of which are important functions in hot environments (Levin, 1973; Karabourniotis *et al.*, 1992; Savé *et al.*, 2000; Hanley *et al.*, 2007). These effects have been found for the North American milkweeds, in which trichome density evolves primarily in response to aridity despite having antiherbivore function (Agrawal *et al.*, 2009b). We also find repeated evolution across *Helianthus* of higher leaf toughness, with shifts to both drier environments and those with a higher diurnal temperature range, and toughness is known to be associated with reduced water loss in drier environments, as well as potentially provide protection against extreme temperatures (Bacelar *et al.*, 2004; Groom *et al.*, 2004; Hanley *et al.*, 2007). Additionally, the resource availability hypothesis was developed to predict investment in herbivore resistance, and may not apply to the less straightforward selective pressures imposed by plant disease. For instance, in wetter environments and those with less seasonal precipitation, we find repeated evolution of higher abundance of the most common secondary metabolites in these defenses.
(HeliaMet Subset PC1). Wetter environments are known to promote the dispersal, colonization, and growth of bacterial and fungal pathogens on leaf surfaces (Fitt et al., 1989; Bradley et al., 2003), and it is possible that higher abundance of hydroxycinnamoyl-quinine derivatives (which have known antimicrobial activity) may prevent infection in wet environments (Zhu et al., 2004; Özcöl et al., 2011). Similarly, we also find repeated evolution of higher lipid content (including resins, waxes, and oils), and higher metabolite profile complexity in more fertile environments. These traits may function in a similar manner if fertile environments promote denser vegetation and a higher ambient pathogen load (Burdon et al., 1989; Freckleton & Lewis, 2006), such that infertile soils provide a refuge from pathogens (Springer, 2009).

Defenses and secondary metabolites predict herbivory and disease resistance

Despite the fact that responses to herbivory and disease were assembled from across a variety of previous screening studies of wild Helianthus, strong relationships were found here between variation in leaf-level defenses and secondary metabolites and...
variation in responses to insect herbivores, fungal pathogens, and a parasitic plant. This suggests that constitutive defenses assessed here play a large role in the response to herbivory and disease across Helianthus, as if differences in resistance among species were primarily attributable to inducible defenses we would not be able to detect the strong patterns observed here. That being said, more work is needed to explore the role of inducible defense across Helianthus, where relatively little work has been done in wild species other than wild H. annuus compared with cultivated sunflower (e.g. Roseland & Grosz, 1997; Prats et al., 2003; Letouze, 2007; Mayrose et al., 2011; Rowe et al., 2012).

Additionally, our results suggest many different defense traits predict resistance to different herbivores and pathogens, providing additional evidence that investment in multiple leaf defenses is not redundant as predicted under the univariate trade-off model. Without strong trade-offs, leaf defense traits can evolve largely independently of one another in response to selective pressure from individual herbivores and pathogens, which may explain in part the high diversity of defenses across Helianthus. Considered in the context of the diverse assemblages of natural enemies probably found across the many habitats occupied by Helianthus species, and the evolution of some defenses in response to the abiotic aspects of these environments, the many combinations of defensive traits observed among Helianthus species is not particularly surprising. This broad diversity of defenses across wild species has been valuable to the improved breeding of cultivated sunflower, resulting in the major successes of breeding Orobanche resistance (identified in Helianthus debilis ssp. tardiflorus) and Sclerotinia stalk rot resistance (identified in several diploid perennials) into cultivated lines (Liu et al., 2012; Velasco et al., 2012). Further expansion of our understanding of adaptation to herbivory and disease in wild Helianthus is likely to remain a valuable avenue to crop improvement.

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Author contributions

C.M.M., A.W.B., B.L.C., R.M.C., C-J.T. and L.A.D. contributed to various aspects of study design. C.M.M. and B.L.C. collected plant tissue and assessed leaf defenses. A.W.B., R.M.C. and C-J.T. collected and analyzed secondary metabolite data. C.M.M., A.W.B. and B.L.C. performed statistical analyses. All authors contributed to the writing of the manuscript.

References


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

Fig. S1 Principal components analysis of the 22 most common HeliaMet compounds.
Table S1  Putative identities and factor loadings for the 22 most common HeliaMet compounds

Table S2  Macroevolutionary correlations between defenses and environmental characteristics

Table S3  Macroevolutionary correlations between defenses and herbivores

Table S4  Macroevolutionary correlations between defenses and pathogens/parasites

Methods S1  Additional methodological details.

Notes S1  Population source localities and USDA accessions, population trait means, species trait means, and herbivore and disease resistance data (data used in this study (Notes S1 and Notes S2) are available via Dryad: doi: 10.5061/dryad.5hq56)

Notes S2  HeliaMet database: normalized peak data obtained by HPLC-MS.

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