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Research

Exploring plant defense theory in tall goldenrod, *Solidago altissima*

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Summary

- Understanding the evolutionary reasons for patterns of chemical defense in plants is an ongoing theoretical and empirical challenge. The goal is to develop a model that can reliably predict how defenses are distributed within the plant over space and time. This is difficult given that evolutionary, ecological, and physiological processes and tradeoffs can operate over different spatial and temporal scales.
- We evaluated the major predictions of two leading defense theories, the growth-differentiation balance hypothesis (GDBH) and optimal defense theory (ODT). To achieve this, enemies, fitness components, terpenoids, and protease inhibitors were measured in *Solidago altissima* and used to construct conventional univariate and structural equation models (SEMs).
- Leaf-tissue value indices extracted from an SEM revealed a strong correlation between tissue value and terpenoid defense that supports ODT. A tradeoff between serine protease inhibition and growth as well as an indirect tradeoff between growth and terpenoids manifested through galling insects supported the GDBH. Interestingly, there was a strong direct effect of terpenoids on rhizome mass, suggesting service to both storage and defense.
- The results support established theories but unknown genotypic traits explained much of the variation in defense, confirming the need to integrate emerging theories such as pollination constraints, defense syndromes, tolerance, mutualisms, and facilitation.

Introduction

Pressure from herbivore and pathogen attack is thought to continuously shape plant defenses in a coevolutionary process (Ehrlich & Raven, 1964). Plants are variously resistant to antagonistic attacks (Painter, 1958) and use an array of strategies including constitutive and induced chemical defenses (Farmer & Ryan, 1990), physical defenses such as spines and thorns (Hanley *et al.*, 2007), and the attraction of mutualistic predators and parasitoids (De Moraes *et al.*, 1998; Kessler & Baldwin, 2001). A continued subject of debate is what drives differential defense allocation patterns in plants. Is defense constrained by other physiological demands such as growth and reproduction? If so, how and at what developmental stages are these demands balanced? At what level of damage does investment in defense provide a net benefit; that is, how tolerant are plants to damage (Hakes & Cronin, 2011)? Here we ask some of these questions in a natural model system, the widely distributed perennial forb *Solidago altissima* (Asteraceae), which is native to North America and invasive in many parts of the world.

Optimal defense theory (ODT) was an early attempt to reconcile the demands of growth and reproduction with those of defense and provided an evolutionary perspective on defense allocation in plants (McKey, 1974; Rhoades, 1979). In its simplest form, it predicts that tissues with the greatest value and vulnerability with respect to fitness (e.g. seeds) should be the most heavily defended. Although optimal defense theory makes some specific predictions, it can be consistent with nearly *any* adaptive defense pattern, making it difficult to falsify (Stamp, 2003). It has performed well in predicting the relative defense levels of highly valued tissues such as flowers (Kessler & Halitschke, 2009) and seeds (Zangerl & Rutledge, 1996), but with respect to tissues with lower direct fitness benefits such as leaves the results are mixed. Alba *et al.* (2012) and a recent meta-analysis offer comprehensive reviews of the evidence in support of ODT with respect to different leaf ages. Both conclude that younger leaves are more valuable and generally more defended than older leaves (McCall & Fordyce, 2010). However, there are cases in which this does not hold. For instance, concentrations of xanthotoxin in leaves of different ages do not vary (Zangerl & Rutledge, 1996) even though there are a number of compelling reasons why younger leaves should be more valuable and vulnerable (McKey, 1974; McCall & Fordyce, 2010). Young tissues are more photosynthetically active and represent a greater investment in future

fitness relative to older tissues, and their higher nitrogen content and relatively undifferentiated state make them more attractive to a range of sucking (Llewellyn & Qureshi, 1978), chewing (Maddox & Root, 1987, 1990), and galling herbivores (Stone & Schonrogge, 2003).

Alternative theories of plant defense include the carbon-nutrient balance hypothesis (Bryant *et al.*, 1983), the growth-rate hypothesis (Coley *et al.*, 1985), and the growth-differentiation balance hypothesis (GDBH) (Loomis, 1953; Herms & Mattson, 1992). The GDBH represents a consolidation of these alternative theories, but remains distinct from optimal defense. It stresses a tradeoff between growth and defense in which differentiating tissues are generally constrained to invest in one or the other, but not both simultaneously. Growth-differentiation balance theories have received moderate support, but numerous studies continue to find no apparent tradeoff between growth and defense (see list in Alba *et al.*, 2012). These four main hypotheses are not mutually exclusive in their predictions and support of a hypothesis may depend on the context and defense trait measured (Barto & Cipollini, 2005).

The aim of this work is unique in that it evaluates the generality across genotypes of the major predictions of optimal defense and growth-differentiation theories of plant defense in a common, perennial, community-dominating forb, *S. altissima* (goldenrod). Goldenrod represents a good model because it is easy to generate genotypic clones with rhizome cuttings. Moreover, the insect community is well described (Root & Cappuccino, 1992), unusually diverse (Messina & Root, 1980), can affect plant fitness significantly (Root, 1996), and has been shown to vary with plant genotype (Maddox & Root, 1987, 1990). In our models, a positive association between tissue value and defense would support ODT. A negative relationship between growth and defense would support the GDBH, regardless of tissue value or risk of attack.

In order to test these predictions, we set out to answer the following questions.

- Which leaf tissues are most valuable to seed (sexual) and rhizome (asexual) reproduction?
- Which leaf tissues are more chemically defended by protease inhibitors and terpenoids?
- How well does leaf tissue value predict chemical defense levels?
- Is there evidence of a tradeoff between plant growth rate and defense?

We focus on protease inhibitors and terpenoids as potential defenses in *Solidago*. Protease inhibitors are costly, resistance-mediating traits in plants against a range of herbivorous insects (Ryan, 1990; Glawe *et al.*, 2003; Zavala *et al.*, 2004) that inhibit gut proteases and thus assimilation of amino acids. This slows the growth and reproduction of herbivorous enemies, but more complex mechanisms have also been proposed (Broadway & Duffey, 1986). In *Solidago*, serine protease inhibitor (SerPI) activity is negatively correlated with generalist caterpillar growth rates and is induced in the plant by caterpillar feeding (Bode *et al.*, 2013).

In *Solidago* foliage, terpenoids are generally stored in internal leaf reservoirs (Anderson & Creech, 1975; Lersten & Curtis, 1989; Curtis & Lersten, 1990) and are among the most metabolically costly plant defenses (Gulmon & Mooney, 1986; Gershenzon, 1989, 1994). They are well known to attract natural enemies, deter herbivore feeding, have alleopathic effects, and inhibit the growth of pathogenic and insect-vectored fungi (Langenheim, 1994). Terpenoids probably represent one of the major defenses in *Solidago* (Johnson *et al.*, 2007, 2010).

Here we used tissue value and plant-height growth rate along with other variables including developmental stage, tissue age, and pesticide treatments to predict defense levels using univariate statistical modeling. We applied pesticides to create variation in herbivore and pathogen levels to assess the possibility of induction and the effects of enemies on plant fitness. However, we found no evidence of induction in this study and thus do not discuss it further. The lack of induction may be attributable to the dominance of galling and sucking insects and the low levels of chewing herbivores observed during the study. We also applied a physio-ecological, exploratory structural equation model (SEM) to this system to evaluate the evidence for ODT and the GDBH and to initiate a broader examination of plant defense theory. In creating an exploratory SEM, we take an initial step toward developing an integrated model of plant defense allocation.

Materials and Methods

Common garden analysis of goldenrod clones

Ten *Solidago altissima* L. clones (i.e. 10.4, 10.6, 10.11, 11.1, 11.5, 11.8, 11.9, 11.12, 12.1 and 12.4) were grown in soilless media (Pro-Mix BX; Premier Tech Ltd, Quakertown, PA, USA) in pots in the glasshouse from rhizomes collected haphazardly from nearby wild populations and transplanted to a common garden at Wright State University (WSU) in June 2008. We transplanted the plants to the field in a 10-row by 10-column grid spaced 2 m on center in a randomized complete block design; each 2-row by 10-column block contained two replicates of each clone. We watered the plants after transplanting, but other than periodical weeding and mowing, they were left to establish on their own. During the summer of 2009 and fall of 2010, we measured plant characteristics in each of the subplots ($n = 10$ subplots per clone) to assess the genotypic status of the 10 clones. These parameters included plant growth rate (rate of height increase); number of ramets at the end of the season; average leaf length to width ratio; average number of leaf teeth cm^{-1} of leaf length; probability of being a cane morph (Wise, 2009); probability of having a red stem; relative flowering time; probability of deer damage; density of crescent, cushion, flat, and irregular gall morphotypes of *Asteromyia carbonifera* (Stireman *et al.*, 2010); average *A. carbonifera* gall growth rates; probability of *A. carbonifera* gall failure; probability of insect meristem damage; density of *Eurosta solidaginis* galls; and the density of *Rhopalomyia solidaginis* galls. A multivariate analysis of variance (MANOVA) was conducted with clone as the predictor. Subsequent ordination of the strongest variables (i.e. $P < 0.10$) was conducted by canonical discriminant analysis using the *candisc* R package (Friendly & Fox, 2013) and 95% confidence ellipses on the mean centers were calculated using established methods (Owen & Chmielewski, 1985).

Testing leaf-tissue value

Additional rhizomes of the same 10 *S. altissima* clones were grown as described in the previous section in the glasshouse and then set out in a deer exclusion plot at WSU (< 400 m from the common garden) on black plastic sheeting in a randomized design (Fig. 1), fertilized once with 5 g of Osmocote® plus (NPK 15-9-12; The Scotts Company, Marysville, OH, USA) at the beginning of the season (26 May 2010) and watered periodically. Each block contained six plants of each clone for a total of 60 plants per block, with eight blocks. Each of two blocks was treated with systemic fungicide (3336 F, thiophanate-methyl; Cleary Chemicals, Dayton, NJ, USA; 1.7 kg AI ha^{-1}), pyrethroid insecticide (Asana® XL emulsible concentrate; esfenvalerate; DuPont™, Wilmington, DE, USA; 75 g AI ha^{-1}), both pesticides, or neither three times during the season (i.e. week of 14 June, 12 July, and 16 August). Within each block, six plants of each clone had either the upper half or lower half of their leaves removed at three different times during the season (early, middle, and late; Fig. 1). The timing of the leaf-removal treatments corresponded to the early pre-branching, branching, and late flowering stages of the plants. One control block with two plants of each genotype (total of 20 plants) was arranged on the edge and did not receive leaf removal treatments, but was treated with both pesticides (Fig. 1). The removed tissue was weighed, placed on dry ice, and transported to a –20°C freezer for storage twice daily while the removal treatments were being conducted.

Leaf tissue value was assessed by measuring capitulum and rhizome mass for leaf-removal treatments and using either coefficients from an SEM or fitness measures as indices of value. Capitula were harvested twice weekly as the pappi began to mature but before they began to disperse. The above-ground biomass was harvested in a single day after all capitula were collected. Rhizomes were separated from the roots and harvested over three consecutive days. Dry masses were determined after oven-drying (55°C) to constant mass.

Protein extraction

Approx. 2 g of frozen leaf tissue from the removal treatments was chopped into 1-cm squares under frozen conditions and re-stored at −20°C. Aliquots were homogenized to powder under liquid nitrogen, transferred to pre-chilled scintillation vials, and stored at −20°C. The powdered samples were freeze-dried in a lyophilizer and re-stored at −20°C. Samples were not allowed to thaw at any stage of processing. Accurately weighed portions (*c.* 50 mg) of powdered, freeze-dried material were vortexed in 1 ml of sodium phosphate extraction buffer (25 mM, pH = 7.0) plus 8.8 mg ml^{-1} NaCl, 2 mg ml^{-1} phenylthiourea (Sigma P5272), 5 mg ml^{-1} sodium diethyldithiocarbamate (Sigma D3506), 2 mM ethylenediaminetetraacetic acid (EDTA; Fisher S80007-1; Fisher Scientific, Pittsburgh, PA, USA), and 50 mg ml^{-1} polyvinylpolypyrrolidone (Sigma P6755), until thoroughly mixed, and centrifuged for 30 min at 16 000 ***g*** and 4°C, and the supernatants were withdrawn and frozen at −20°C until analysis.

Protein assays

Stored extracts were centrifuged for 15 min as described in the previous section to pellet any residual material before taking two

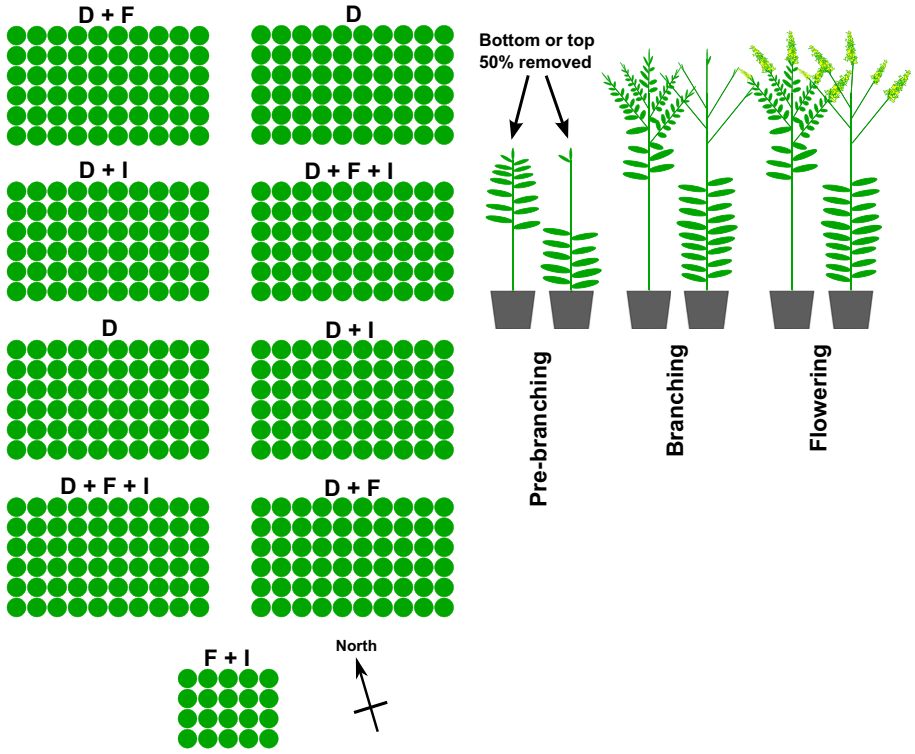

Fig. 1 Experimental plot layout and diagram of leaf defoliation treatments. Each block contained 2 × 3 × 10 plants to allow the defoliation treatments to be applied separately to individual potted plants over 10 *Solidago altissima* genotypes. To allow relatively normal growth, the flower buds or apical meristems were not removed. Either the mature or young leaf tissue was removed from pre-branching, branching, or flowering plants. All blocks, except the small control subplot, had defoliation (D) treatments. Various subplots had fungicide (F; thiophanate-methyl), insecticide (I; esfenvalerate), or both (F + I) applied at three evenly spaced time-points during the growing season. There were also two replicates each of nine of these genotypes grown in the glasshouse (not illustrated).

technical replications per sample for total protein and per cent serine and cysteine protease inhibition (SerPI and CysPI, respectively) assays. Samples and plates were kept on ice during loading. Total protein assays followed the protocol of Bradford (1976) with bovine serum albumin as a standard. We measured both SerPI and CysPI levels in the removed tissues with established methods adapted for use in a microplate reader system (Abe *et al.*, 1994; Bode *et al.*, 2013).

For SerPI activity, 20 µl of 0.1 M Tris-HCl (pH = 8.0), 10 µl of trypsin (Sigma T8003; 0.25 mg ml^{-1} in Tris-HCl), and 20 µl of soybean trypsin inhibitor (Sigma T9003; as a positive control) or sample were added to each well. Blanks had trypsin replaced with Tris-HCl and negative controls had sample replaced with extraction buffer. The covered plate was shaken for 3 s and incubated for 5 min at 37°C. Then 20 µl of N-benzoyl-DL-arginine-β-naphtylamide (BANA; Sigma B4750; 3.1 mg ml^{-1} in dimethyl sulfoxide) was added and the plate incubated at 37°C for 20 min. Then, 100 µl of 2% HCl in ethanol was added to each well to stop the reaction and the background absorbance was read at 540 nm. Finally, 100 µl of p-dimethylaminocinnamaldehyde (p-DACA; Sigma D4506; 0.6 mg ml^{-1} in ethanol) was added, and the plate was shaken, incubated at room temperature for 120 min, and then read at 540 nm. Cysteine protease activity was measured similarly except that papain (Sigma P4762; 0.25 mg ml^{-1} in 25mM sodium phosphate buffer; pH = 7.2) replaced trypsin and reaction buffer (250 mM sodium phosphate buffer, pH = 6.0, plus 2.5 mM EDTA) replaced the Tris-HCl. Preliminary experiments indicated that the CysPI activity was saturated at the sample concentration; therefore, the CysPI assays were conducted with a 1 : 20 dilution of the sample extract. Milli-Q water (18.2 M ohms) was used for all solutions. Protease activity was expressed as per cent inhibition relative to the negative control:

$$I = (c - x)100c^{-1} \quad \text{Eqn 1}$$

(x, corrected absorbance of the sample reaction; c, corrected absorbance of the negative control.) All absorbances were corrected by subtracting the background absorbance of the well before adding dye. Finally, the mean of the two technical replications per sample was divided by the mean sample protein concentration.

Terpenoid extraction

To *c.* 50 mg of accurately weighed freeze-dried leaf tissue, 7.5 µg of tetralin (internal standard) was added and the tissue was extracted in 1.8 ml of methanol for 24 h at room temperature. The extracts were centrifuged for 2 min at 830 ***g*** and the supernatant was transferred to glass vials to which 1.5 ml of HPLC-grade hexanes and 200 µl of water were added. After another 24 h at room temperature, the organic layer was run over hexane-preconditioned, silica-gel columns (0.20 g) and stored at −20°C until analysis by gas chromatography–mass spectrometry (GC-MS).

GC-MS analysis

GC-MS analysis was conducted on a Varian CP-3800 GC and a Saturn 2000 mass detector (Varian Saturn 2200 GC-MS-MS unit; Agilent Technologies, Santa Clara, CA, USA) run by Varian MS WORKSTATION v6.6 software. In general, analysis followed the method described in Johnson *et al.* (2010). One microliter of each sample was injected through a Varian 1079 injector, which was maintained at 250°C. Gas flow was initially set at splitless for 0.5 min; subsequently the split ratio was set at 100 for 4.5 min and then maintained at 30 for the rest of the run. For compound separation, we used a Varian® Factor Four capillary column VF-5 ms (30 m × 0.25 mm ID, DF = 0.25). The initial column oven temperature was set at 50°C (4-min hold), was then increased to 130°C at 8°C min^{-1} (1.0-min hold) and finally was increased to 280°C at 10°C min^{-1} (12-min hold). The column flow rate was set at 1.0 ml min^{-1}. The MS transfer line temperature was held at 250°C, the ion trap at 150°C and the manifold at 50°C while ionization voltage was maintained at 70 e/v. Quantification was performed by expressing the signal intensity of individual peaks relative to that of the internal standard tetralin. Major ion peaks were inspected using Varian® MS WORKSTATION, DATA REVIEW v6.6 SP-1. Structures of terpenoids were confirmed based on capillary GC retention times (Johnson *et al.*, 2010) and mass spectral comparisons with the NIST 2004 database.

Modeling trends in fitness components and defense levels

The effects of leaf age and plant stage as well as the fungicide and insecticide treatments on fitness and defense were assessed with a fully nested mixed model with block and genotype as random effects. The model was run with the lme function in R statistical software (R Core Team, 2013) using the *nlme* package (Pinheiro *et al.*, 2013). The models were all specified exactly the same for each of the responses. The responses were final dry capitula mass, final dry rhizome mass, final above-ground biomass (minus the removed tissue), growth rate (the slope of the line connecting the sum of stem heights at the pre-branching and branching stages), per cent SerPI, per cent CysPI, and total mono-, sesqui-, and diterpenoids (Supporting Information Methods S1). Some missing values in the data set occurred because of vole damage and early senescence of mature leaves; however, a series of imputation procedures indicated that this minor imbalance did not substantially affect the conclusions.

Modeling defense theory predictions

To test how well components of ODT and the GDBH predict defense levels, the same starting linear model was used to predict each of the defense measures. The effects of capitula mass, rhizome mass, growth rate, fungicide and insecticide treatments, age, and stage were assessed as well as all two-way interactions with a fully nested mixed model with block and genotype as random effects (Methods S1). These full models were subsequently reduced to the final models with the stepAIC function in the R package *MASS* (Venables & Ripley, 2002). The lme output

does not contain the sum-of-squares needed to calculate the per cent of variation explained by each of the predictors. Therefore, a fixed effects model based on the reduced model provided by the stepAIC function with and without genotype included was used to obtain sum-of-squares.

Structural equation modeling

An SEM is uniquely suited for analyzing relationships where predictors may conceivably act as both independent and dependent variables. Conventional univariate and multivariate techniques cannot deal appropriately with such data (Grace, 2006; Kline, 2011), and path analysis entails a restrictive set of assumptions (e.g. no measurement error). In plants, for example, above-ground biomass is clearly associated with seed production, but it can also be influenced by herbivory or pathogen attack.

In an SEM, the specification of a path should be justified theoretically or experimentally. Before it was possible to fully specify the SEM, it was first necessary to determine the direction of the path between galling insects and growth rate, because either direction is plausible; that is, galling insects may be more attracted to genotypes with higher growth rates (Horner & Abrahamson, 1992) or they may directly influence growth rates via hormonal mechanisms (Impson *et al.*, 2013; Tewari *et al.*, 2013). Two separate and largely independent analyses were used to accomplish this. The first was an SEM with genotype effects included. In this SEM, we specified paths from genotypes to galling insects, above-ground biomass, and growth rate as well as a feedback loop from galling insects to growth rate to above-ground biomass and back to galling insects. In a second analysis we regressed the mean growth rates of the genotypes grown in the absence of galling insects (i.e. in the glasshouse) on the mean number of galls found on these genotypes per stage in the field. Because mean growth rate by genotype in the glasshouse and field were strongly positively correlated and because galling insects began attacking plants immediately after they were placed in the field (data not shown), a positive relationship in this regression would suggest attraction of galling insects to genotypes with higher growth rates.

To elucidate potential indirect evidence for ODT or the GDBH, we specified an exploratory SEM. We specified reciprocal paths between growth rate and all four defense measures because the GDBH predicts that growth should compete with defense and vice versa (Herms & Mattson, 1992). We also specified directional paths from purely exogenous variables (i.e. stage × age and ducking) to all endogenous variables (i.e. the four defense measures, growth rate, final above-ground biomass, number of chewing, sucking, or galling insects, rust fungus, capitula mass, and rhizome mass). To control for potential effects of above-ground biomass on herbivore abundance, a path was specified from above-ground biomass to each herbivore group. Two separate analyses indicated that galling insects increased plant growth rates; therefore, this path was also specified. Herbivores were also specified to affect rust fungus, capitula mass, and rhizome mass. All the defense measures were specified to affect all other endogenous variables, but not each other. Rust fungus was specified to affect above-ground biomass, growth rate, capitula mass, and rhizome mass. Aside from the covariances associated with the dummy coded exogenous variables, three additional covariances were specified between sucking and galling insects, between capitula and rhizome mass, and between terpenoid richness and total terpenoids. The specification of a covariance between sucking and galling insects and between terpenoid richness and total terpenoids accounted for residual correlations in preliminary models. The covariance between capitula and rhizome mass was theoretically specified based on the assumption of a tradeoff between vegetative and sexual reproduction (Grime & Pierce, 2012).

To stabilize variances, terpenoid richness, total terpenoids, and chewing, sucking, and galling insects were $\log_e(x+1)$ transformed; SerPI and CysPI were $\log_e(x+100)$ transformed; and rust fungus (i.e. area under the disease progress curve) was $\log_e(x+40)$ transformed. The SEM analyses were run in the R package *OpenMx* (Boker *et al.*, 2011) and the full R code is provided (Methods S1, Table S1). The amounts of variation explained (i.e. R^2 values) were calculated as blocked-error R^2 (beR^2) using the procedures described in Hayduk (2006) to avoid the influence of one slightly inflated disturbance estimate.

Results

Goldenrod clones

Ordination revealed at least eight distinguishable phenotypes among our 10 *S. altissima* clones as determined by nonoverlapping 95% confidence ellipses on the means in at least one perspective (Fig. 2a–c). Clones 11.1, 11.12 and 12.1 were not resolved as phenotypically unique by the first three canonical axes, which described a total of 80.8% of the variation. However, principal components analysis of the terpenoids revealed that clone 12.1 was completely isolated in ordination space (data not shown), indicating the presence of at least nine phenotypically distinguishable clones, referred to hereafter as genotypes.

Leaf tissue value

Measuring the effect on fitness of removing leaf tissues of various ages and at different stages of plant development allowed us to assess the value of those leaf tissues. Removal of the young tissue at the branching stage reduced capitula mass the most (Fig. 3b, stage: df = 2, 152; $F = 16.5$; $P < 0.001$; age: df = 1, 228; $F = 26.2$; $P < 0.001$; stage × age: df = 2, 228; $F = 4.4$; $P = 0.013$), but the effect of tissue removal on rhizome mass was weak. Only the age of the tissue removed affected rhizome mass, and this was only evident in an interaction with fungicide treatment (Fig. 3d; fungicide: df = 1, 4; $F = 23.0$; $P = 0.009$; fungicide × age: df = 1, 228; $F = 4.3$; $P = 0.039$) in this univariate analysis. As expected, removing any type of leaf tissue reduced fitness in terms of both capitula mass (compare Fig. 3a,b with c) and rhizome mass (compare Fig. 3d with e). For practical reasons, the nonremoval treatments were not randomly mixed in with the main experimental blocks (Fig. 1), and therefore one has to make these comparisons with caution. However, this design would have no impact on the utility of the removal treatments for comparing tissue value.

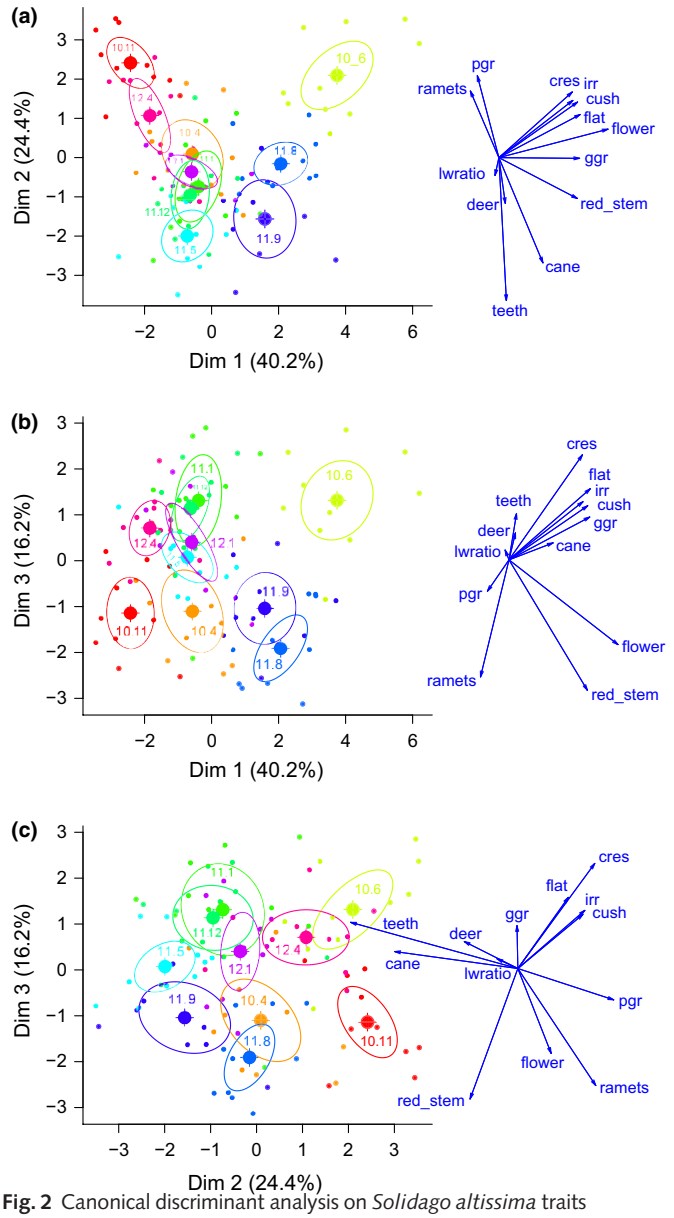

Fig. 2 Canonical discriminant analysis on *Solidago altissima* traits measured on 10 clones grown in the ground in a common garden. Vectors indicate the direction of increase in the various traits measured: number of ramets (ramets), plant growth rate (pgr), density of *Asteromyia carbonifera* morphotype galls (crescent, cres; irregular, irr; cushion, cush; flat); flowering time (flower); average *A. carbonifera* gall growth rate (ggr); probability of red stem (red_stem); probability of exhibiting the 'ducking' morphotype (cane); average number of leaf teeth per cm (teeth); probability of deer damage (deer); leaf length to width ratio (lwratio). The vector origins actually start at (0,0), but are shifted for clarity. Large solid circles indicate ellipse centers. Note that clones 11.1, 11.12 and 12.1 were not resolved with 95% confidence ellipses on the means in at least one perspective and therefore may represent the same genotype, but ordination of the terpenoid data clearly differentiates 12.1 (data not shown).

Protease inhibition and terpenoids

Leaves on young branching plants had the highest levels of SerPI, which then declined (Fig. 4a; stage: df = 2, 150; $F = 14.8$; $P < 0.001$; stage × age: df = 2, 212; $F = 9.1$; $P < 0.001$), while CysPI peaked in leaves of mature branching plants, and then declined slightly thereafter (Fig. 4b; stage: df = 2, 150; $F = 48.1$; $P < 0.001$). CysPI was slightly higher in mature tissue (Fig. 4b; age: df = 1, 212; $F = 27.1$; $P < 0.001$) and the application of fungicides had minor but significant effects that depended on the removal treatments (Fig. 4b; fungicide × stage: df = 2, 150; $F = 3.5$; $P = 0.034$; fungicide × stage × age: df = 2, 212; $F = 3.7$; $P = 0.025$).

Regardless of stage, young leaves had higher concentrations of terpenoids than old leaves (Fig. 5a,b). Leaves on young branching and young flowering plants had the highest concentrations of monoterpenoids (Fig. 5a; stage: df = 2, 88; $F = 148.1$; $P < 0.001$; age: df = 1, 126; $F = 298.4$; $P < 0.001$; stage × age: df = 2, 126; $F = 20.3$; $P < 0.001$) and monoterpenoids peaked at the branching stage and remained high thereafter (Fig. 5a). Sesquiterpenoids in leaves peaked at the branching stage, but declined slightly thereafter (Fig. 5c; stage: df = 2, 88; $F = 59.0$; $P < 0.001$; age: df = 1, 126; $F = 146.0$; $P < 0.001$; fungicide × insecticide × stage: df = 2, 88; $F = 4.3$; $P = 0.017$). There were no significant effects on diterpenoids (see Figs S1–S17 for boxplots that include all factors and responses).

Predicting defense levels

We used capitula mass, rhizome mass, growth rate (which varied among genotypes; Fig. 6), pesticide treatments, stage, and age to predict defense levels. Table 1 shows the results of four linear mixed models, one for each defense measure. Overall, genotype, stage, age, and tissue value explained most of the variation in defense levels. Tissue value explained 7.4%, 1.2%, and 9.5% of the variation in SerPI, CysPI, and total terpenoids, respectively. None of the variation in terpenoid richness was explained by either capitulum or rhizome mass. Surprisingly, growth rate was only a significant predictor for SerPI when it interacted with capitula mass and even then only explained 0.6% of the variation. Growth rate explained 1.7% of the variation in CysPI. Stage and age were among the strongest predictors of all the defenses, except age on SerPI. Overall, genotype explained most of the variation (11–52%; Table 1). Likelihood ratio tests of the models with and without genotype included indicated that genotype was an extremely important and significant predictor ($P < 0.0001$ for all defenses).

The exploratory structural equation model

Both independent analyses indicated that galling insects increase the growth rates of plants. First, a separate SEM incorporating the effects of genotype on growth rate and galling insects indicated a significant positive relationship between galling insect abundance and growth rate (Fig. 7a). Secondly, the growth rates of genotypes grown in the glasshouse, which lacked galling insects, were not associated with galling insect abundance measured on the same genotypes in the field (Fig. 7b).

The full SEM is presented in Fig. 8 with only significant paths shown (i.e. $P \leq 0.05$). Figs 9 and 10 present subsets of the paths

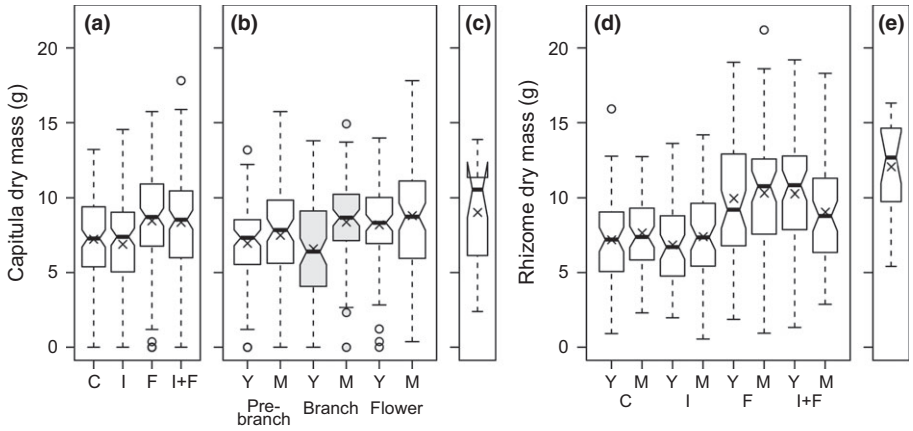

Fig. 3 Box and whisker plots for *Solidago altissima* capitulum (a–c) and rhizome (d, e) dry mass for significant factors. Box notches approximate a 95% confidence interval. The '×' is the mean and the median is the horizontal bar. Circles represent outliers. (a) Capitula dry mass over different pesticide treatments including a negative control (C), insecticide treatment (I), fungicide treatment (F), and both insecticide and fungicide (I+F) treatment. (b) Capitula dry mass when 50% of young (Y) or mature (M) tissue was removed at the pre-branching, branching, or flowering stage. (c, e) Capitula (c) or rhizome (e) dry mass for the small control subplot, which did not receive leaf-tissue removal treatments but did receive both pesticide treatments. (d) Rhizome dry mass over various pesticide treatments and where either young or mature tissue was removed. Boxes at the branching stage are shaded for clarity.

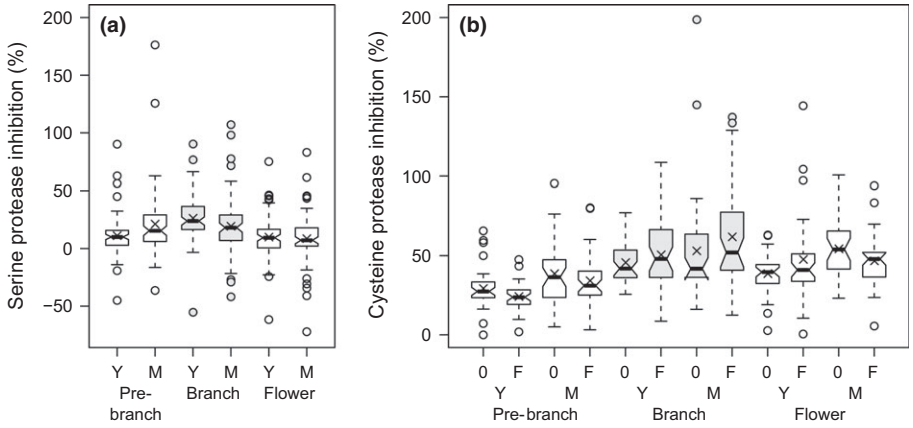

Fig. 4 Box and whisker plots for *Solidago altissima* per cent serine (a) and cysteine (b) protease inhibition for significant factors. Box notches approximate a 95% confidence interval. The '×' is the mean and the median is the horizontal bar. Circles represent outliers. (a) Per cent serine protease inhibition for young (Y) or mature (M) leaf tissues removed at the pre-branching, branching, or flowering stage. (b) Per cent cysteine protease inhibition for young or mature leaf tissues removed at the pre-branching, branching, or flowering stage and either treated (F) or not (0) with fungicide. Boxes at the branching stage are shaded for clarity.

from Fig. 8 to focus attention on specific areas of the model that support either ODT (Fig. 9) or the GDBH (Fig. 10).

The age × stage factor (Figs 8, 9) was coded relative to the most valuable leaves, namely those on young branching plants; therefore, a *positive* (i.e. solid arrow) coefficient indicates one of three things depending on the path. If the path is directed toward a defense measure then it means that the defense levels were higher in that stage × age tissue than they were in the young branching stage. If the path is directed toward a fitness measure then it means that removing that stage × age leaf tissue had less of an impact on fitness than removing young branching leaf tissue (i.e. it decreased fitness less). If it is directed toward an enemy then it means the enemy was more abundant on plants having had that stage × age leaf tissue removed than it was on plants having had their young branching leaf tissue removed. All SEM paths are standardized and thus in units of standard deviations. Overall, removal of any stage × age leaf tissue resulted in significantly higher fitness (i.e. rhizome mass) than removing leaves of young branching plants. Thus, leaves of young branching plants are the most valuable (Fig. 9). CysPI was lowest at the pre-branching stage and had similar levels at other stages. Terpenoid richness was lowest at the pre-branching mature and flowering mature stages. Plants that had their young leaf tissue removed at the flowering stage had a slightly higher abundance of galling insects than those that had young leaves removed at earlier stages (Fig. 8). Rust fungus was highest in those plants that had their young tissue removed at the pre-branching stage (Fig. 8).

To assess the correspondence between tissue value and defense levels, the unstandardized coefficients for the paths leading from the stage × age variables to the defense measures were regressed

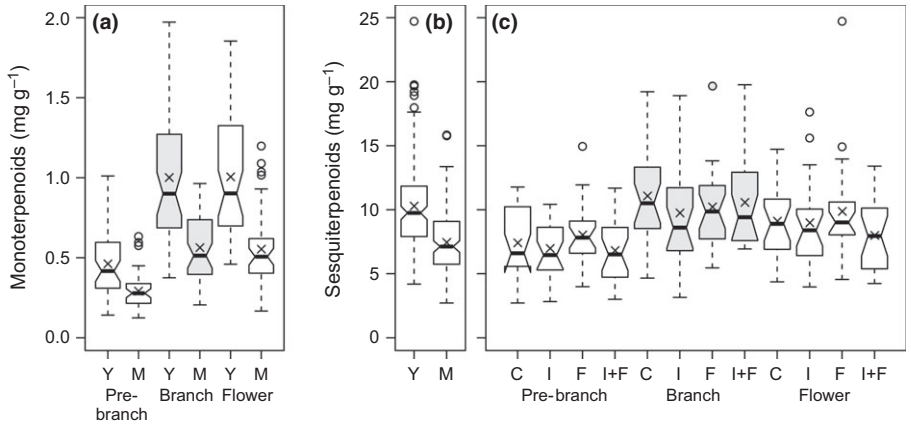

Fig. 5 Box and whisker plots for *Solidago altissima* mono- and sesquiterpenoids (mg g^{-1} dry leaf tissue) for significant factors. Box notches approximate a 95% confidence interval. The '×' is the mean and the median is the horizontal bar. Circles represent outliers. (a) Monoterpenoids in young (Y) or mature (M) leaf tissues removed at the pre-branching, branching, or flowering stage. (b) Sesquiterpenoids in young (Y) or mature (M) leaf tissue pooled over plant stage. (c) Sesquiterpenoids in leaf tissues removed at the pre-branching, branching, and flowering stages and treated with no pesticides (C), insecticide (I), fungicide (F), or both (I+F). Boxes at the branching stage are shaded for clarity.

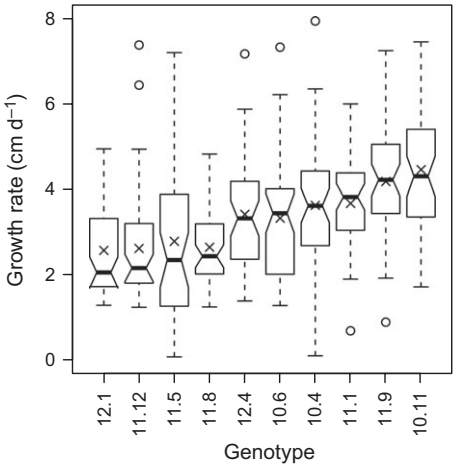

Fig. 6 Box and whisker plots for plant growth rates (cm d^{-1}) for the 10 *Solidago altissima* genotypes used in this study. Box notches approximate a 95% confidence interval. The '×' is the mean and the median is the horizontal bar. Circles represent outliers.

against the corresponding coefficients for paths leading from the stage × age variables to either capitula mass or rhizome mass. All eight regressions were run, but only the significant ones are shown (Fig. 9b,c). There was a strong significant correlation between tissue value and terpenoid richness and between tissue value and total terpenoids (Fig. 9b,c).

The SEM included reciprocal paths from growth rate to all the defense measures, but only the direct path to SerPI was significantly negative (Fig. 10). The remaining paths are not shown because none was significant in either direction (Table S2). However, there was a moderately significant tradeoff between growth rate and defense, which manifested itself as an indirect effect through galling insects (Fig. 10).

Some *S. altissima* genotypes express a 'ducking' trait where the apex of the growing stem is bent in the shape of a walking cane (see Wise, 2009). In the SEM this dichotomous 'ducking' trait had significant effects throughout the model, including direct and indirect positive effects on fitness as well as direct negative effects on rust fungus (Fig. 8).

Finally, total terpenoids was strongly positively associated with rhizome mass. This was a serendipitous finding associated with the process of constructing the exploratory SEM. In addition to the indirect path through herbivores, this effect suggests a direct effect of terpenoids on rhizome mass.

Discussion

We tested the degree to which the major predictions of ODT and the GDBH of plant defense allocation were supported in the constitutive defenses of the perennial herb *S. altissma*. We found strong support for ODT and moderate support for the GDBH. Leaf tissue value in terms of vegetative reproduction (rhizome mass) was a strong predictor of leaf terpenoid concentrations and richness. Growth rate was negatively associated with terpenoid concentrations and richness, but only indirectly through their negative effects on galling insects. Growth rate was also negatively associated with SerPI, but this explained relatively little variation.

Variation in leaf tissue value

Leaf tissues of different age and developmental class (i.e. stage) clearly have different value with respect to fitness, but leaf tissue value was a better predictor of defense levels when it was assessed in terms of rhizome mass than capitula mass. In the SEM, removal of branching young tissue reduced rhizome mass the most; however, in the univariate analysis age interacted with pesticide treatment and stage was not significant. The reason stage was not significant in this analysis was that the effect of total terpenoids on rhizome mass was not included. The reason fungicide treatment was significant in the univariate analysis is unclear. Fungicide was applied to create variation in rust fungus, which had only minor effects on rhizome mass in the SEM, clearly too small to account for the larger effects seen in the univariate analysis. Perhaps the fungicide altered resource allocation toward

Table 1 Statistics for the nested mixed effect models aimed at predicting chemical defense levels in *Solidago altissima* genotypes[1]

	Num. df	Den. df	*F*	*P*	% explained[2]	% explained[3]
Serine protease inhibition						
Capitula	1	218	36.0	<0.001	7.4	7.4
Growth rate	1	218	0.7	0.415	0.1	0.1
Stage	2	156	12.1	<0.001	4.3	4.3
Age	1	218	2.6	0.106	0.4	0.4
Genotype	9	–	–	–	19.7	NA
Capitula × growth rate	1	218	8.3	0.004	0.6	3.4
Stage × age	2	218	6.8	0.001	1.8	1.8
Residuals	444				65.8	82.8
Cysteine protease inhibition						
Capitula	1	220	5.6	0.019	1.2	1.2
Growth rate	1	220	11.4	0.001	1.7	1.7
Fungicide	1	6	0	0.981	0	0
Stage	2	156	43.9	<0.001	14.7	14.7
Age	1	220	26.5	<0.001	3.3	3.3
Genotype	9	–	–	–	11.4	NA
Capitula × fungicide	1	220	8.3	0.004	0.9	1.1
Residuals	445				66.8	78.0
Terpenoid richness						
Stage	2	94	8.2	0.001	2.5	NA
Age	1	137	27.0	<0.001	4.2	NA
Genotype	5	–	–	–	52.4	NA
Residuals	273				41.0	NA
Total terpenoids						
Rhizomes	1	136	15.8	<0.001	9.5	NA
Stage	2	94	73.1	<0.001	16.9	NA
Age	1	136	180.5	<0.001	20.7	NA
Genotype	5	–	–	–	21.7	NA
Residuals	272				31.2	NA

[1]The same starting model included capitula mass, rhizome mass, growth rate, fungicide and insecticide treatments, stage, age, and all two-way interaction terms. Age was nested in stage which was nested in genotype which was nested in block. Each starting model was reduced by applying the stepAIC function in R. The Bayesian information criterion (BIC; $k = \log_e(n)$) was used for model selection. Num., numerator; Den., denominator.
[2]Per cent of variation explained = factor sum of squares divided by total sum of squares from a linear fixed effect model including genotype as a fixed effect.
[3]Per cent of variation explained from a linear fixed effect model not including genotype. NA, not applicable.

rhizomes. With respect to capitula mass, young branching leaf tissue was the most valuable in both analyses. This echoes earlier findings. Mid-stage, young leaf tissue appears most valuable across herbaceous plant families, including *Arabidopsis thaliana* (Brassicaceae; Barto & Cipollini, 2005) and *Nicotiana sylvestris* (Solanaceae; Ohnmeiss & Baldwin, 2000).

Growth rate as a predictor of defense levels

SerPI was negatively related to growth rate in the SEM, suggesting a tradeoff between these factors and support for the GDBH. However, growth rate was unrelated to the other defense measures except indirectly through galling insects. This is surprising, as terpenoids are generally found in a highly reduced form in plants, which makes them costly chemical defenses (Gershenzon, 1994). Plant strategy models have been proposed that may, in part, explain this discrepancy. Grime & Pierce (2012) propose the CSR (competitive, stress-tolerant, ruderal) model which classifies plants and other organisms in ordination space according to tradeoffs between: growth and reproduction, reproduction and stress tolerance, and stress tolerance and growth. Perhaps *S. altissima* is positioned closer to the tradeoff between growth and stress tolerance and further from the tradeoff between growth and reproduction. Growth rate was not significantly influenced by measured variables in univariate analyses, save genotype. Perhaps our measure of growth rate was not sufficient to capture the fine details of leaf expansion. If this is the case, then these differences would be captured in the 'final above-ground biomass' variable, which was indeed negatively related to terpenoid richness in the SEM (Fig. 8). This final point lends additional support to the GDBH.

Tissue value as a predictor of defense

At first glance it would appear that tissue value is a good predictor of defense levels, because there is some correspondence between the tissues of highest value (i.e. when removed fitness is reduced) and the defense levels of those same tissues. However, closer inspection of this correspondence reveals that it does not hold across life stages. For instance, while capitula mass was reduced substantially by removal of the youngest tissues (pre-branching stage), defense levels were among the lowest at this stage (Fig. 3b). This may be the result of a tradeoff. At the early stages of development, winning the competitive fight for resources is

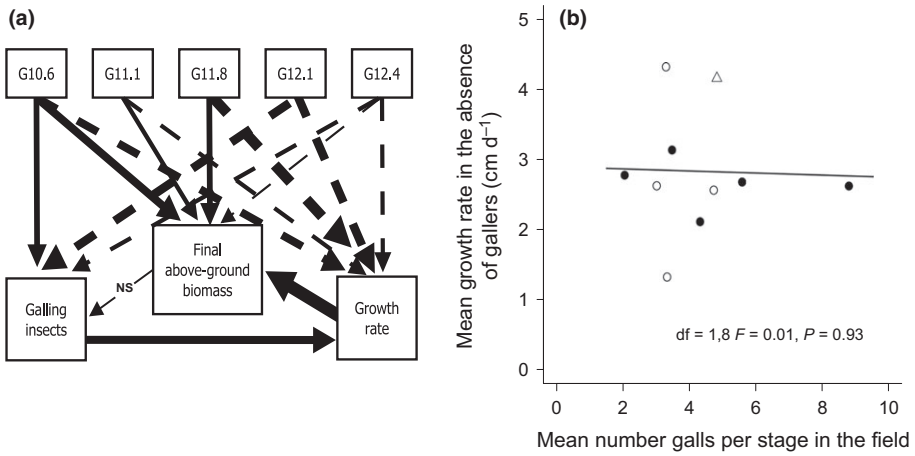

Fig. 7 Two different analyses of the effect of galling insects on *Solidago altissima* growth rate. (a) Structural equation model with genotype effects included to control for the potential confounding effects of insect attraction to specific genotypes with high growth rates. Path widths are proportional to their corresponding standardized coefficient. Solid and dashed paths indicate positive and negative coefficients, respectively. Only significant (i.e. $P \leq 0.05$) paths are shown. NS, not significant. For clarity, variances, genotype covariances, and disturbance estimates are not shown. (b) Linear regression of mean genotype growth rate in the absence of galling insects (i.e. glasshouse-grown genotypes) on the mean number of galls per stage in unprotected (i.e. field-grown) genotypes. The line is the relationship for those genotypes used in the SEM analysis (closed circles). Genotype 11.9 (triangle) was used in the SEM analysis but was not grown in the glasshouse; therefore, it has a field growth rate. The open circles are genotypes also grown in the glasshouse, but not included in the SEM analysis. Regardless of which set of points is used, the relationship is not significant.

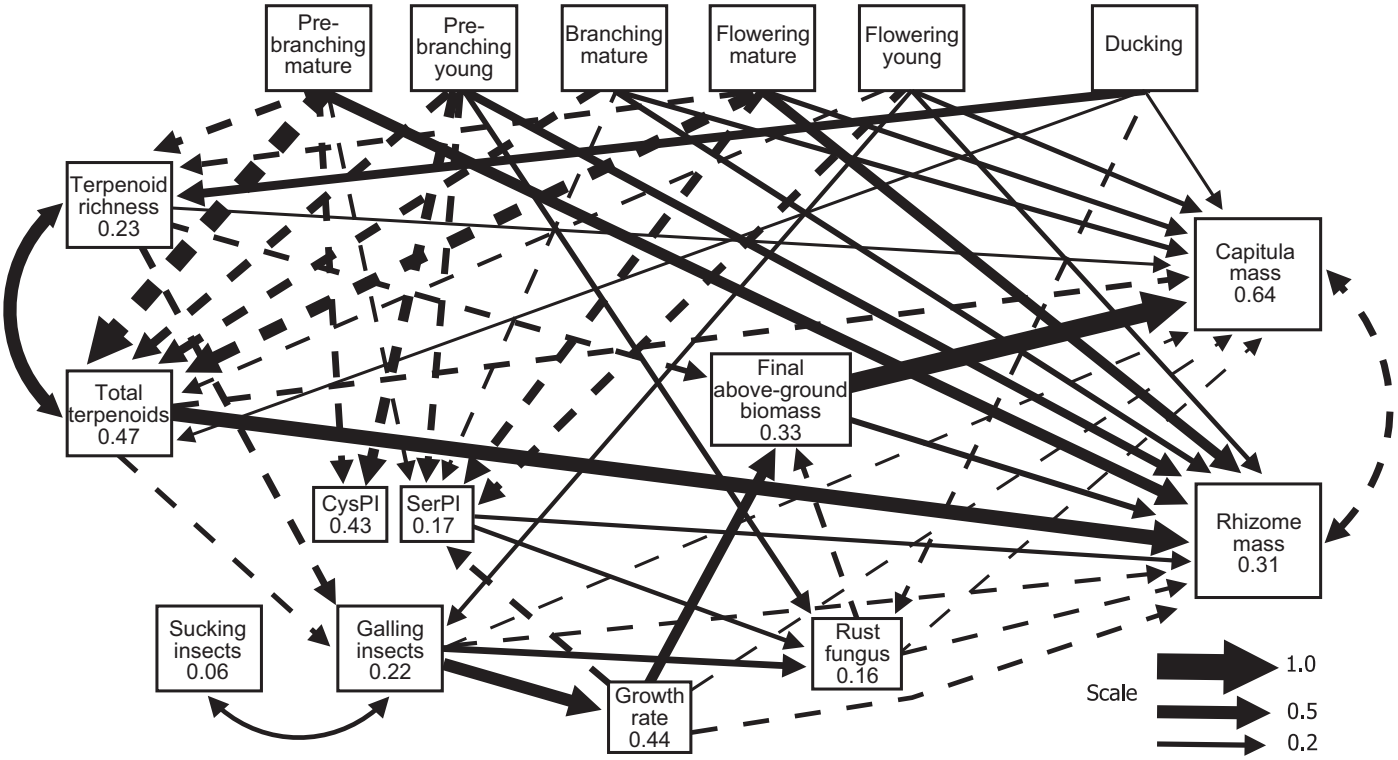

Fig. 8 Structural equation model for a pooled subset of six *Solidago altissima* genotypes where a complete set of chemical defense data was available. Path widths are proportional to their corresponding standardized coefficient. Solid and dashed paths indicate positive and negative coefficients, respectively. Only significant (i.e. $P \leq 0.05$) paths are shown, but all specified paths were retained. All covariances and variances were specified for the purely exogenous dummy coded variables but are not shown for clarity. This is also true for endogenous disturbances. The numbers below the variable names are blocked-error R^2 (beR^2) values and indicate the proportion of variation explained. Model statistics: $n = 282$; df = 3; $\chi^2 = 2.3$; $P = 0.51$; Comparative fit index (CFI) = 1.00, Tucker–Lewis Index (TLI) = 1.03.

paramount across phyla (Simberloff, 1982). If chemical defense synthesis draws resources away from growth, or growth and defense are constrained, then one would expect lower investment in defense during this stage. The GDBH predicts as much (Herms & Mattson, 1992). When all factors are considered together in an SEM, estimates of tissue value are more accurate

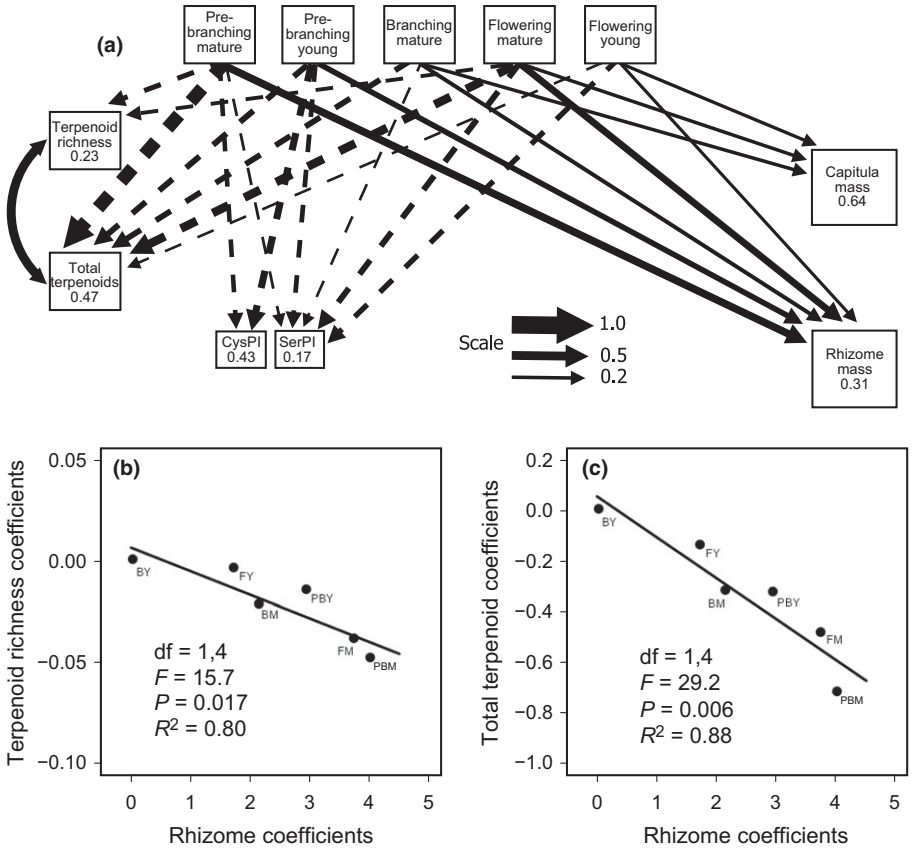

Fig. 9 (a) A subset of paths from the full *Solidago altissima* structural equation model that support optimal defense theory. This is the portion of the full structural equation model (see Fig. 8) showing only the paths from the stage × age dummy coded variables. Note that the stage × age effect is coded relative to the most valuable stage × age (i.e. young branching leaf tissue) and therefore this stage × age level is not shown. For example, a positive coefficient from 'pre-branching mature' to 'rhizome mass' indicates that when that tissue was removed rhizome mass was higher than when it was removed from the young branching stage. Similarly, a negative path from 'pre-branching mature' to 'total terpenoids' indicates that total terpenoids were lower in the 'pre-branching mature' tissue than they were in young branching leaf tissue. Therefore, young tissue is more defended and more valuable. (b, c) Significant correlations between the raw path coefficients leading to fitness measures (i.e. an index of tissue value) and the level of chemical defense in those tissues. Each point is labeled with its corresponding stage × age level: pre-branching stage (PB), branching (B), flowering (F), young (Y), and mature (M). Note that all eight regressions were run, but only the significant ones are shown.

and a strong correspondence between tissue value and defense is revealed, but only when rhizome mass is the proxy for fitness. These results strongly support ODT for leaf tissues with respect to asexual reproduction, but only weakly or not at all for sexual reproduction. For perennial plants with the potential for vigorous asexual reproduction, producing seeds may be more of an escape mechanism or a means of purging deleterious mutations than a reproductive strategy. In this sense, one might expect rhizome mass to be a more accurate measure of tissue value and thus correlate more strongly with defense levels.

Terpenoids as both defenses and storage molecules

Levels of SerPI, CysPI and sesquiterpenoids decreased in the later stages of development as did the fitness values of leaf tissue, but monoterpenoids and CysPIs remained high. Terpenoid biosynthesis in plants generally begins as the leaves are expanding. Biosynthesis and storage in various secretory structures continue until the leaves are fully expanded, at which point they slow or stop. There is little turnover in healthy plants until flowering, at which time some terpenoids are glycosylated and may be transported to rhizomes (Croteau *et al.*, 1984; Gershenzon, 1989, 1994). It is likely that the decrease in defenses at the flowering stage as well as the strong positive effect of total terpenoids on rhizome mass in the SEM is associated with cost-saving catabolism. In fact, the GDBH suggests that tissues may be expected to avoid the tradeoff between growth and defense if stored resources can be used for growth (Herms & Mattson, 1992) and optimal defense would predict that the least useful (in terms of defense) would be catabolized first. Monoterpenoids did not decrease in the flowering stage, which suggests that they may be more important in late-stage leaf defense than sesquiterpenoids in this system, but studies aimed at quantifying the fitness benefits in terms of rhizome mass of catabolized terpenoids are needed to fully address the biological significance of this effect.

Intraspecific variation in defense and ecological costs

Perhaps our most notable result, given that conventional defense theory does not generally and explicitly embrace genotypic

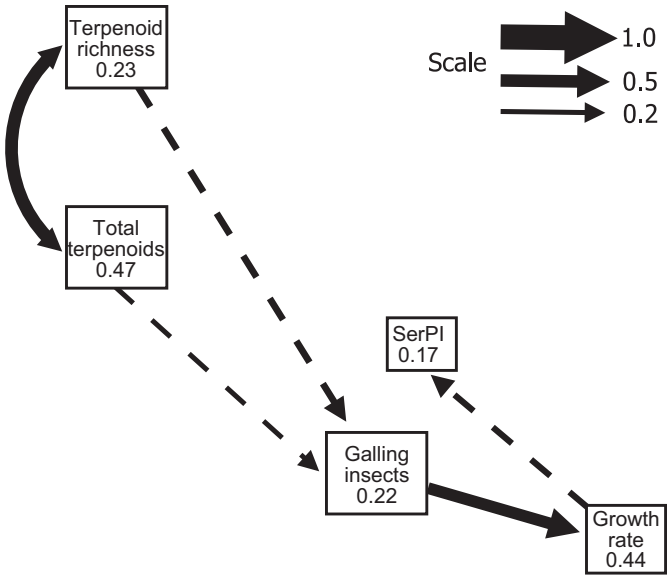

Fig. 10 A portion of the full *Solidago altissima* model (see Fig. 8) with only the paths that support the growth-differentiation balance hypothesis shown for clarity. Galling insects significantly increase growth rates (see also Fig. 7) and terpenoids decrease galling insects; therefore, there is a degree of indirect tradeoff between growth and terpenoid defense. A significant direct negative relationship also exists between per cent serine protease inhibition (SerPI) and growth rate.

variation, is the degree to which genotype predicts defense levels despite inclusion of variables expected to explain genotypic differences. Genotype was by far the most important predictor of variation overall. This may be explained by specific ecological strategies employed by different genotypes, as was suggested by Maddox & Root (1987, 1990). They showed that different *S. altissima* genotypes appeared resistant (*sensu* Painter, 1951) to a specific 'suite' of herbivores. Thus, natural selection may favor and maintain strategies that allow colonization of specific ecological and environmental microhabitats even within plant species. Understanding the maintenance of such strategies in a population will probably require applying inclusive fitness theory (Karban *et al.*, 2013) or balancing selection models (Bradshaw & Schemske, 2003; Delph & Kelly, 2014).

Even though terpenoids can be considered costly defenses, they still require only a small fraction of the entire energy budget of the plant to produce (Gould, 1983; Gulmon & Mooney, 1986; Gershenzon, 1994). Perhaps the majority of the costs of terpenoid production are ecological. Interestingly, one of the genotypes (11.5) in this study which is the most resistant to the fungus-vectoring gall midge *Asteromyia carbonifera* (data not shown) and had the highest levels of SerPI was heavily attacked by voles in our experiment. The voles did not appear to eat the foliage, but rather chopped entire stems up into material that they used to build nests in the same potted plants. We have also seen this under natural field conditions, suggesting that it is a common occurrence and a potential cost to high levels of chemical defense. Harpy eagles are known to line their nests with fresh green foliage, perhaps to repel pest insects (Seymour *et al.*, 2010), and these voles may be using a similar strategy which results in an ecological cost to *S. altissima* chemical defense.

Pollination constraints may also select against high levels of defense. This would be especially true for an insect-pollinated, obligate outcrosser such as *S. altissima*. In a wild tomato species (*Solanum peruvianum*), caterpillar-damaged plants have higher concentrations of specific mono- and sesquiterpenoids in the flower volatiles that reduce pollination and consequently plant fitness (Kessler & Halitschke, 2009; Kessler *et al.*, 2011).

Conclusions

Our results suggest that at least two more layers of defense theory are needed (in addition to ODT and the GDBH) to effectively explain the variation in plant defense allocation. One would incorporate life history traits mainly associated with strategies evolved to cope with environmental perturbations. Isoprene, for instance, is important in plants for dealing with drought and heat stress (Loreto *et al.*, 2004; Sharkey *et al.*, 2008) and its production may tradeoff with the production of larger terpenoids used mainly for defense against enemies or as fuel to increase rhizome or root mass as the photosynthetic capacity of the leaves decreases during late-season senescence. In short, some plant compounds may serve both defense and storage functions, which may obscure tradeoffs when only fitness endpoints are measured. The strong direct path in the SEM between terpenoids and rhizome mass supports this.

Another layer of theory is needed to account for ecological idiosyncrasies associated with suites of herbivore pressure, pollination syndromes, mutualistic associations with natural enemies, and perhaps facilitation imposed by herbivores. Much of this work has already begun. Defense syndromes (Agrawal & Fishbein, 2006), constraints imposed by plant defense on multitrophic interactions (Poelman *et al.*, 2008), and evolutionary dilemmas (van der Meijden, 1996) are all in line with this thinking. However, a more holistic theory of plant defense will require integrating established and emerging theory (Alba *et al.*, 2012).

Identifying the factors that explain differences across genotypes will guide the development of an integrated model of plant defense. A variety of strategies may coexist within species, with each being maintained by spatially and temporally varying selection pressures. Future empirical studies of plant defense should use an individual SEM to each genotype, but with sufficient sample sizes to fit individual SEMs to each genotype. They should also include additional factors associated with drought, nutrients, tolerance, and ecological peculiarities. It may well be that the most effective strategy for a plant species is a variable one (Denno & McClure, 1983).

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Supporting Information

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

Figs S1–S17 Boxplots for all responses and factors.

Table S1 Covariance matrix needed as input to run the SEM code

Table S2 Coefficients, standard errors, and *P* values for all SEM paths

Methods S1 R code for the SEM and univariate mixed models.

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