Nicotine has a minor role in protecting field-grown *Nicotiana benthamiana* against insect herbivory

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Abstract

Plants produce an immense diversity of defensive specialized metabolites. However, despite extensive functional characterization, such experiments are seldom performed in natural settings, and the balance between different defensive compounds is rarely examined. Here, we compare the efficacy of three *Nicotiana benthamiana* defensive compounds, nicotine, acylsugars, and a serine protease inhibitor, by growing plants with combinations of knockout mutations in a natural setting, quantifying invertebrate interactions, and comparing relative plant performance. Among the three tested compounds, acylsugars had the greatest defensive capacity, affecting aphids, leafhoppers, spiders, and flies. Nicotine mutants displayed increased leafhopper feeding and aphid colonization. Plants lacking both nicotine and acylsugars were more susceptible to flea beetles and thrips. By contrast, knockout of the serine protease inhibitor did not affect insect herbivory in the field. Complementary experiments under controlled laboratory conditions confirmed results obtained in a natural setting. We conclude that the three metabolite groups, which provide defense against different herbivores and herbivore classes, collectively provide broad-spectrum protection to *N. benthamiana*. However, there is a gradient in their effectiveness, with acylsugars providing the strongest protection and nicotine providing a secondary line of defense. Furthermore, we demonstrate that, even if individual metabolites do not have a measurable defensive effect on their own, they can have an additive effect when combined with other defensive compounds.

Introduction

Plants synthesize an enormous diversity of specialized metabolites, with estimates ranging from 200,000 to 1,000,000 unique compounds in the plant kingdom (1). Any individual plant produces roughly 1,750 – 3,500 specialized metabolites with diverse functions (2). Many families of specialized metabolites, such as benzoxazinoids (3), cardiac glycosides (4), and glucosinolates (5), have well-documented functions in plant defense, whereas others are yet to be investigated. All plants produce multiple classes of specialized metabolites with differing defensive functions. For instance, *Erysimum cheiranthoides* (wormseed wallflower) accumulates both cardenolides and glucosinolates (6) and *Nicotiana attenuata* (coyote tobacco) produces nicotine (7), chlorogenic acid (8), protease inhibitors (9) and volatile terpenes (10), which each have a distinct protective role. Furthermore, the effects of the different defensive metabolites are not only additive, but also can involve more complex interactions. Examples include a protective function only in the presence of additional metabolites (11), reciprocal reduction in toxicity (12), or co-option by specialist insect herbivores for their own protection (13, 14). Therefore, altering the production of several defensive compounds simultaneously and examining their ecological impacts in a natural setting are essential for gaining insight into their functions and interplays in plant defense.

Biosynthesis of nicotine, a pyridine alkaloid built from the conjugation of a pyridine ring and a nitrogen containing pyridinium ring (15), is restricted to *Nicotiana* species (16). The function of nicotine in protecting plants against insect herbivores has been extensively demonstrated under both laboratory (17, 18) and field conditions (7). Nicotine functions primarily by targeting nicotinic acetylcholine receptors in animal nervous systems (19), and investigations of tritrophic interactions exemplify the complex relation between this specialized metabolite, insects that consume it, and their predators (20, 21).

Acylsugars are a diverse family of compounds with the common feature of a sugar core that has several aliphatic chains of different lengths connected to it (22). These metabolites, which are secreted from glandular trichomes of plants in the Solanaceae and several other families (23–25), have an inhibitory effect on insect herbivores in controlled insect-plant interaction studies, both *in planta* (26–29) and *in vitro* (30). Several studies have identified acylsugar biosynthesis genes in tomato and other Solanaceae (25, 31, 32), paving the way for targeted manipulation of these defenses.

Protease inhibitors are proteins that inhibit insect digestive enzymes, rendering the consumed plant material less nutritious (33). These plant proteins, which are induced in response to caterpillar feeding, jasmonate treatment, or wounding (34), reduce caterpillar growth (9, 35), slow aphid development (36), and reduce attractiveness of plants to mirids (9). Field-grown

Solanum nigrum (black nightshade), in which expression of three serine protease inhibitors was silenced, was more susceptible to both thrips and caterpillars (37).

Nicotiana benthamiana is an Australian tobacco species that adapted to desert conditions millions of years ago (38). This species is widely used for plant molecular biology research, largely due to its extreme viral susceptibility (39) and amenability for transient gene expression assays (40). By contrast, very few laboratory studies involve the investigation of insect interactions in this species, likely due to the high level of endogenous herbivore resistance.

In the current study, we utilized existing genome-edited *N. benthamiana* plants and generated additional mutants and mutant combinations to examine the function of three representative defensive compounds, nicotine, acylsugars, and a serine protease inhibitor, in both a natural setting and controlled laboratory conditions. We found, that acylsugars had the strongest effect, nicotine had a comparatively moderate effect, and the protease inhibitor had no discernible effect on invertebrate interactions.

Results

Generation of a622 and asat2 mutants

To generate *N. benthamiana* lines with reduced nicotine and acyl sugar content, the *A622* gene, which is key to nicotine production (41), and *ASAT2*, which is essential for acyl sugar production (42), were targeted for CRISPR mutagenesis. Plants were infected with tobacco rattle virus (TRV) carrying guide RNAs (gRNAs) targeting either the *A622* gene, or a combination of the *A622* and *ASAT2* genes in both *CAS9*-expressing wildtype and *CAS9*-expressing *serp2* mutants, which do not produce the Serp-II protease inhibitor (43). We assessed foliar nicotine and acylsugar content in the next generation using LC-MS (Fig. S2). Multiple independent lines from both backgrounds and infiltrated constructs contained less than 5% of normal nicotine abundance (Fig. S2A). When analyzing acylsugar abundance, we found phenotypic mutants in the *CAS9* background, but no plants with less than 5% acylsugar abundance in the *serp2* background. However, there was one line close to the 5% threshold whose progeny were grown for further examination (Fig. S2D, F).

When confirming the mutations using Sanger sequencing, we found that the *A622* gene often was missing stretches of DNA between the three gRNA targets (1-3, 1-2, or 2-3), but single-basepair indels also were formed (Fig. S4). The *ASAT2* gene displayed a range of mutations, including 136, 29, 3 and 2-base pair deletions (Fig. S5). Based on these results, additional plants were grown and screened (Fig. S2, S3) until the final panel for field experiments was assembled. This panel did not include the *serp2+a622+asat2* triple mutant, which was still being characterized and would be included later in controlled laboratory experiments.

Effects of nicotine, acylsugars and SERP2 on invertebrate interactions in a natural setting

The mutant population was planted in a plowed 20 x 10 m field section, in 24 blocks of 14 plants (Fig S1) and invertebrate interactions were observed at five time points up to day 59 post-planting

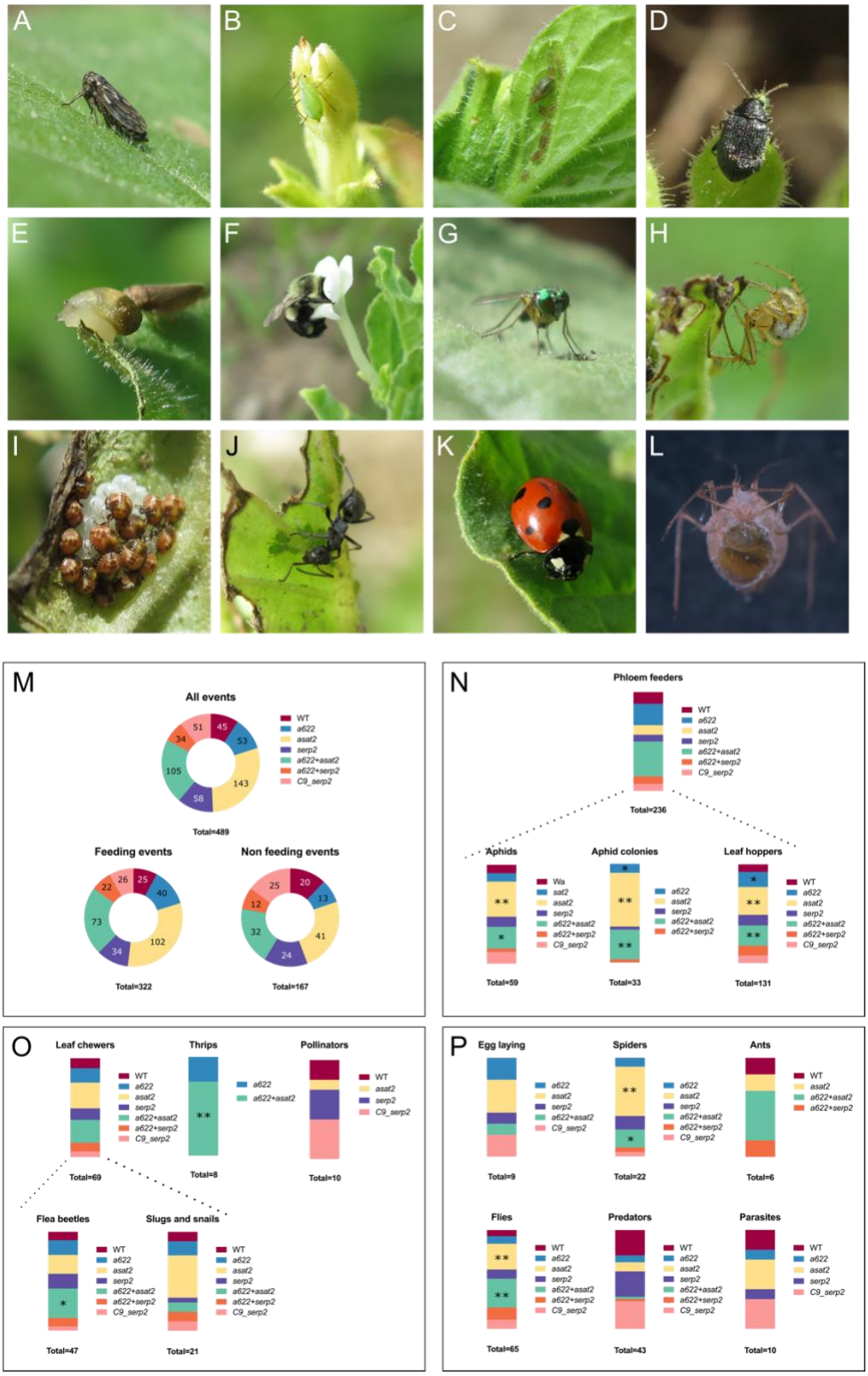

Figure 1. Invertebrate interactions observed in a natural setting. (A-L) Photographs of invertebrates comprising the main interactors observed in the field. (M-P) a breakdown of the interaction frequencies. (**A)** Leafhopper from the genus *Agallia*. (B) Leaf aphid (C) Leaf aphid colony (D) Flea beetle from the *Epitrix* genus. (E). Snail, probably from the genus *Succinea*. (F) Bumble bee (genus *Bombus*) pollinating a *N. benthamiana* flower. (G) Fly from the genus *Condylostylus*. (H) An orb weaver spider, probably from the species *Mangora acalypha*. (I) Stink bug (*Halyomorpha halys*) eggs during hatching. (J) Ant "farming" an aphid colony. (K) Lady bug (*Coccinella septempunctata*). (L) Parasitized aphid collected from *N. benthamiana* with a wasp larva that has almost entirely consumed the aphid's body. (M) All the observed events separated to feeding and non-feeding interactions. Feeding interactions are further divided to (N) phloem feeders, (O) leaf chewers and, with thrips and pollinators not falling strictly in either of those categories. (P) Non feeding interactions. These include all interactions in which invertebrates interacted with the plant but did not directly feed on it. Within each invertebrate type, frequencies were compared across the plant treatments by a Chi-square test with Benjamini-Hochberg correction (*$p<0.05$, **$p<0.01$).

(Fig. 1, S6; Supplemental Data 1). Throughout the wet summer months, snails and slugs caused the greatest amount of plant damage. During the first observation cycle (4-7 d post planting) many leafhoppers and flies but no aphid colonies were observed. During the second cycle six (18-23 d post planting), aphid colonies appeared and fewer flies and more than double the previous number of flea beetles were observed. During the third observation cycle (31-34 d post planting), aphids and aphid colonies tripled, despite fewer plants being monitored due to high mortality in the first row closest to the field margins. Far fewer leafhoppers and flea beetles were observed during this cycle, but spiders tripled. A new predator, an assassin bug, appeared on eight plants, totaling 10 individuals. At this point bumble bees pollinated plants, which had substantial numbers of flowers. During the fourth observation cycle (45 d post planting), fewer plants were monitored. However, aphids and aphid colony numbers were maintained whereas other herbivore numbers were reduced. Assassin bug numbers increased to 29 individuals, whereas spiders maintained their numbers. An additional observed interaction was four instances of aphids parasitized by wasps. Finally, the fifth observation cycle (59 d post planting) included only three blocks. Despite this small number, six parasitized aphids were observed, as well as five plants with whiteflies, which were not seen in the first four observation cycles.

When analyzing all of the collected data, which included over 600 individual invertebrates observed on plants (Fig. 1M), we observed clear effects of some of the mutations. Leafhoppers, by far the most commonly observed insects (131 events totaling 187 individual insects), were found more frequently on *asat2*, *a622*, and *a622+asat2* mutants (Fig. 1N), demonstrating that

both nicotine and acylsugars protect plants from leafhoppers (Fig. S7A). Aphids were found on significantly more *asat2* and *a622+asat2* mutants (Fig. 1N), indicating that acylsugars but not nicotine are important for protection against these phloem feeders (Fig. S7B). In contrast to this, aphid colonies were found more often on *asat2*, *a622*, and *a622+asat2* plants, and none were found on wildtype plants (Fig. 1N), indicating that despite not affecting the individual aphids, nicotine does affect aphids' ability to colonize *N. benthamiana* (Fig S7C). Flea beetles, the most abundantly observed leaf chewers (Fig. 1O), were significantly more abundant on *a622+asat2* mutants, indicating that both acylsugars and nicotine provide protection, and plants only become susceptible when losing both defenses (Fig. S7D). Similar results were obtained for thrips, which were found more commonly on *a622+asat2* mutants. Slugs and snails showed a trend of being found on more *asat2* mutants. Bumble bee pollinator visitations were statistically similar between all plants due to the low numbers observed (Fig. 1O), However, the bees seemed to prefer plants that had more flowers regardless of their chemotype. In non-feeding interactions (Fig. 1P), flies and spiders preferred *asat2* and *a622+asat2* mutants. When contemplating whether spiders prefer *asat2* mutants due to higher prey abundance or lower irritation upon contact, we turned to our observation that orb weavers, which often wove their webs between an *asat2* mutant and another line, were usually found on the *asat2* mutant, indicating that they preferred to stay on those plants.

Insect predators, including lady bugs and assassin bugs, were found significantly less commonly on *a622+asat* and *a622+serp* plants. However, this is most likely due to the different plant sizes, as plant height at the end of the experiment correlates significantly with average assassin bug abundance on the different chemotypes (R^2=0.83, p=0.0042). Three other observation classes, ants, egg-laying, and parasites, did not show differences between the mutants due to their rare occurrence rate (6-10 events). However, it is interesting to note that the ratio of parasitized aphids to aphid and aphid colony occurrence, is not correlated (R^2=0.17, p=0.35 and R^2=0.04, p=0.68 respectively). This lack of correlation could indicate that wasps parasitize aphids with greater ease when aphids are on better-protected plants.

We next examined the statistical relation between *asat2*, *a622*, and *a622+asat2* mutants to better understand when effects seen in double mutants were additive, and whether one of these specialized metabolites had a stronger effect than the other. We compared seven interactions in which some of these mutants were significantly different from wildtype. There was no difference between the three mutants in leafhopper, flea beetle, and thrips interactions, indicating that both nicotine and acylsugars protect plants similarly from these insects. In the case of aphids, aphid colonies, and flies, both *asat2* and the double mutants were significantly different from nicotine mutants, indicating that only acylsugars protect plants from aphids and flies and that their protection from aphid colonization is stronger than nicotine to the extent that nicotine has no

added effect on this interaction when acylsugars are affected. Spiders were affected only by acylsugars, but by neither nicotine nor the double mutants, indicating a combined effect of acylsugars and plant size.

When examining interactions in the field, we noticed that the *asat2+serp2* mutants did not show similar interactions as the other *asat2* and *a622+asat2* mutants. Indeed, when examining the nicotine and acylsugar content of representative field grown plants, we found that both independent lines, which originated from different parents, did not have a reduced acylsugar content (Fig. 2B). We next sampled the remaining plants from these genotypes in the field to ascertain whether this was a segregating phenotype and found that all the *asat2+serp2* plants sampled had an acylsugar abundance above the 5% threshold compared to wildtype (Fig. S8). Despite this surprising result, we kept these genotypes in the analysis, since these plants are still mutated in their *serp2* gene, and refer to them as *C9_serp2* hereafter.

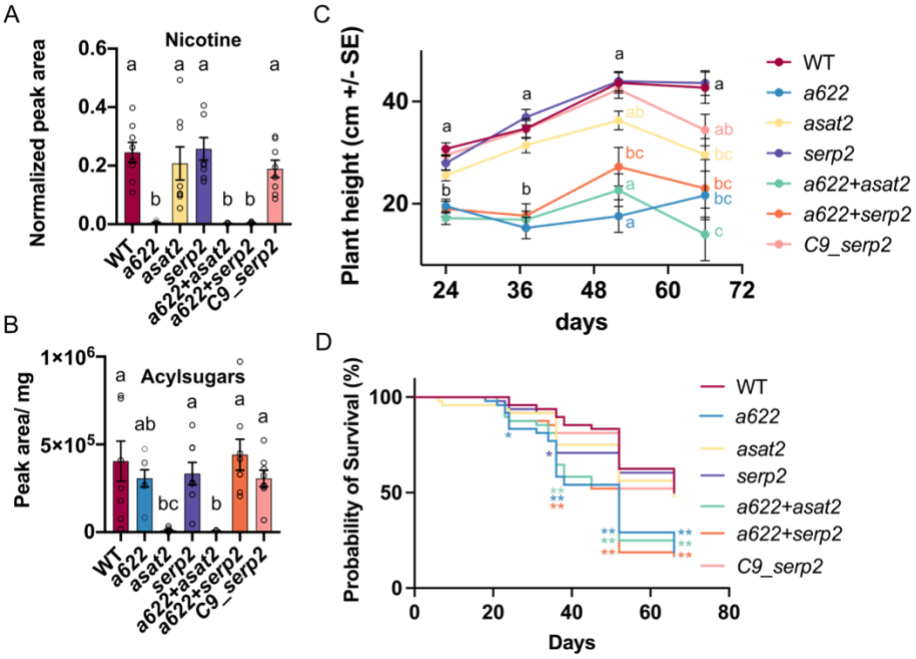

Figure 2. Nicotine and acylsugar abundance of field-grown plants, plant height, and survival. (A) Nicotine abundance determined by LC-MS with peak normalization by a bipyridine internal standard and tissue weight. (B) Acylsugar abundance determined by LC-MS with the peak area normalized to tissue weight. (C) Height of field grown plants throughout the growing season. Dead plants (as scored by lack of stem greenness) were removed from the analysis. Different letters indicate significance of $p < 0.05$ as determined by Tukey's HSD test. Results in A-C are presented as the mean ± SE. (D) Survival frequency of field grown *N. benthamiana*. *$p < 0.05$ **$p < 0.01$. as determined in a Chi-square test, comparing wildtype and each mutant.

When examining general fitness parameters, we monitored plant height and survival. In general, all lines that harbored mutations in the *A622* gene were smaller (Fig. 2C). However, this is an *a622*-specific phenotype, which also can be seen when growing *a622* mutants under controlled conditions. For this reason, plants moved to the field were grown under conditions that enabled size matching prior to field planting. In addition to the effects of the *a622* gene, at the last measurement point *asat2* mutants were significantly smaller than wildtype, displaying a cumulative effect of the increased invertebrate susceptibility. *Serp2* plants were similar to wildtype at all time points. In addition to height, plant survival was assessed (Fig. 2D). Here we found that all plants harboring the *a622* mutation survived at lower rates, whereas mutations in *asat2* did not affect plant survival. Seed production could not be assessed because, due to the transgenic status of the plants, capsule development needed to be stopped at early stages.

Insect interactions under controlled conditions

Interactions observed in the field are the sum of many different factors combined into a single phenotype. Therefore, we next examined the two main feeding guilds, phloem feeding and leaf

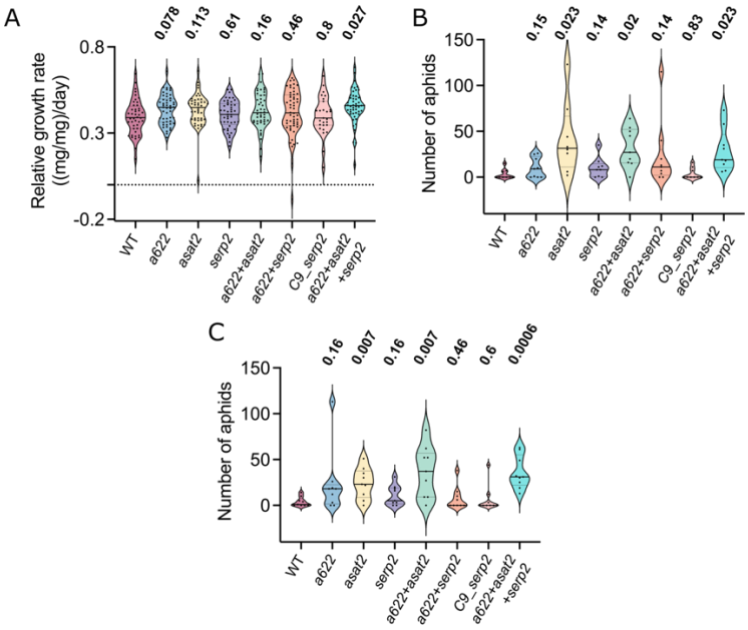

Figure 3. *Chloridea virescens* growth and *Myzus persicae* reproduction under controlled conditions. (A) *C. virescens* weight following four days of feeding on *N. benthamiana* leaves. Larvae were placed in mesh bags on individual leaves four days post-hatching. (B-C) The number of aphids in leaf cages, 8 (B) or 9 (C) days following the removal of all but three newly born nymphs. (B) Non-nicotine-tolerant *M. persicae* strain. (C) Nicotine-tolerant *M. persicae* strain. P values of

student's *t*-tests are displayed above mutants that were compared to wildtype, with a Benjamini-Hochberg correction. Solid lines represent median values. Dashed lines represent quartiles.

chewing, under controlled conditions. We initially examined the interaction of the broad generalist caterpillar *Spodoptera exigua* (beet armyworm) using a population that included the triple *a622+asat2+serp2* triple mutant. However, only 11 caterpillars out of 390 neonates placed on 199 plants from the eight genotypes survived, and no trend was seen of higher survival on plants that had fewer defensive compounds. Therefore, we performed an additional trial with *Chloridea virescens* (tobacco budworm), with caterpillars 4 days post-hatching, rather than first-instar larvae. Of the seven mutant combinations examined, only the triple *a622+asat2+serp2* mutants had significantly heavier caterpillars (Fig. 3A). This result is intriguing since the *serp2* mutants showed no effect in field and controlled conditions, but the addition of the *serp2* mutation beyond the *a622+asat2* mutations lead to caterpillars being heavier than on wildtype.

We next examined the effects of our mutant population on aphid reproduction using both nicotine-sensitive and nicotine-adapted *M. persicae* strains. Results were identical for the two strains, with aphids reproducing faster on mutants containing an *asat2* knockout (Fig. 3B-C). All other mutant combinations were similar to wildtype. Just as when the plants were grown in the field, acylsugars dominated defense against aphids, and the other compounds were less important in this context.

An additional question we wished to address, was whether aphid preference of plants harboring *asat2* mutations in the field was due to an increased "choice" of these plants, increased growth and survival of the aphids, or a combination of the two. We therefore performed dual choice assays using leaf discs. Twenty-one chemotype combinations were examined using the two aphid strains. We found that the nicotine-adapted strain, which did not show a different response compared to the nicotine sensitive strain in the reproduction assay, did not significantly prefer any of the chemotypes, indicating a broader tolerance to chemical defenses (Fig. S9). However, when all leaves that have an *asat2* mutation were compared against those that do not, a significant preference for the *asat2* mutants was revealed (Fig. S9W). Surprisingly, aphids from the "nicotine susceptible" strain did not show a preference for nicotine-free leaves (Fig. 4E, I, H, M, N, O, Q). Furthermore, when comparing all leaves containing normal nicotine abundance to those where nicotine was reduced, aphid preference was similar (Fig. 4V). By contrast, all leaves harboring the *asat2* mutation were preferred compared to wildtype (Fig. 4B, D, F), and also relative to many of the other mutants (Fig. 4G, K, L, N, P, R, S, U). The combination of all *asat2* mutants compared to all leaf discs not harboring an *asat2* mutation, was significant as well (Fig. 4W).

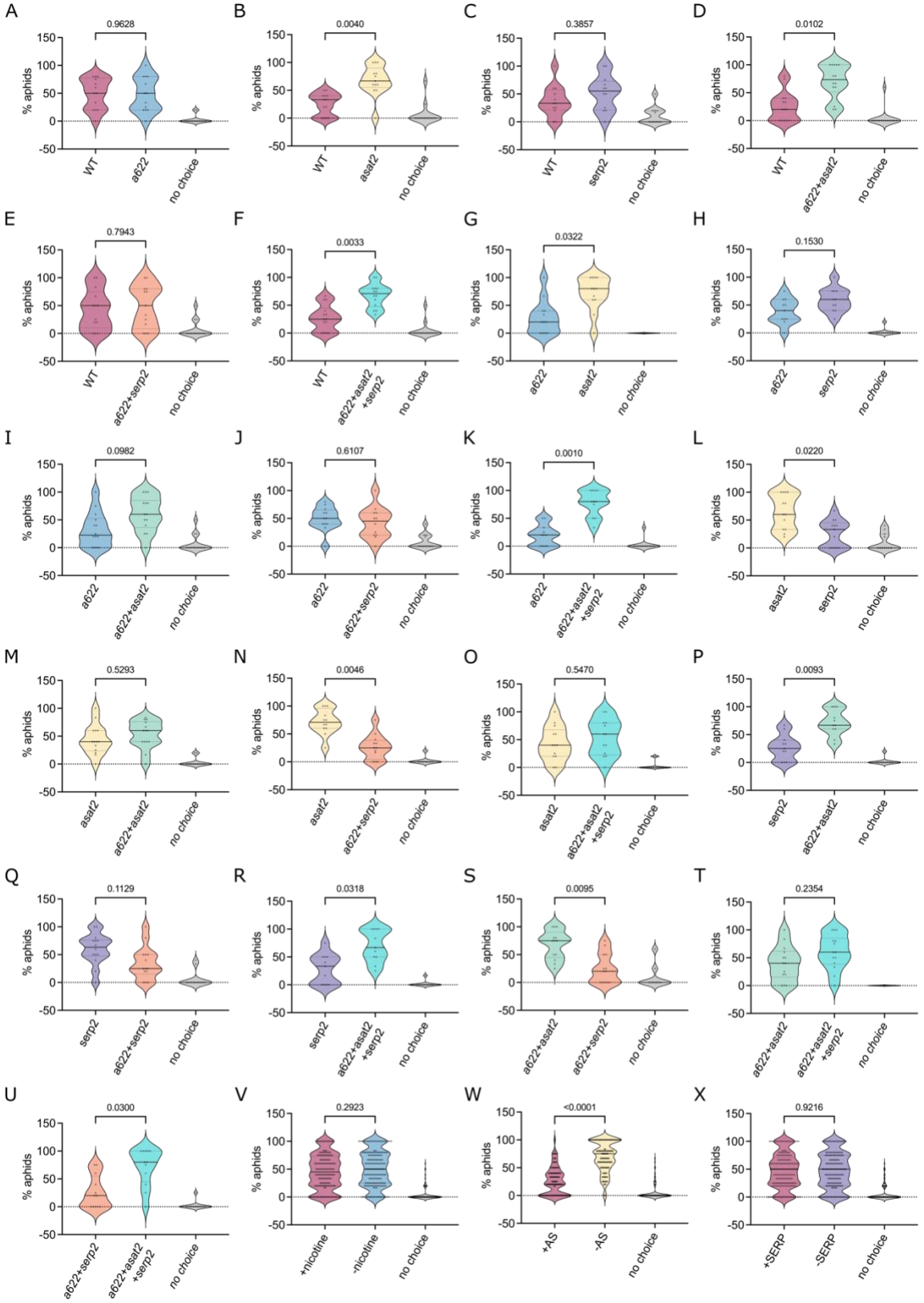
A
0.9628
% aphids
WT
a622
no choice
B
0.0040
% aphids
WT
asat2
no choice
C
0.3857
% aphids
WT
serp2
no choice
D
0.0102
% aphids
WT
a622+asat2
no choice
E
0.7943
% aphids
WT
a622+serp2
no choice
F
0.0033
% aphids
WT
a622+asat2 +serp2
no choice
G
0.0322
% aphids
a622
asat2
no choice
H
0.1530
% aphids
a622
serp2
no choice
I
0.0982
% aphids
a622
a622+asat2
no choice
J
0.6107
% aphids
a622
a622+serp2
no choice
K
0.0010
% aphids
a622
a622+asat2 +serp2
no choice
L
0.0220
% aphids
asat2
serp2
no choice
M
0.5293
% aphids
asat2
a622+asat2
no choice
N
0.0046
% aphids
asat2
a622+serp2
no choice
O
0.5470
% aphids
asat2
a622+asat2 +serp2
no choice
P
0.0093
% aphids
serp2
a622+asat2
no choice
Q
0.1129
% aphids
serp2
a622+serp2
no choice
R
0.0318
% aphids
serp2
a622+asat2 +serp2
no choice
S
0.0095
% aphids
a622+asat2
a622+serp2
no choice
T
0.2354
% aphids
a622+asat2
a622+asat2 +serp2
no choice
U
0.0300
% aphids
a622+serp2
a622+asat2 +serp2
no choice
V
0.2923
% aphids
+nicotine
-nicotine
no choice
W
<0.0001
% aphids
+AS
-AS
no choice
X
0.9216
% aphids
+SERP
-SERP
no choice
150
100
50
0
-50

Figure 4. Non nicotine-adapted *Myzus persicae* choice between 21 combinations of two leaf discs of wildtype and mutant plants. (A-U) comparison of single lines. The compared lines are indicated below the panels. (V-X) A comparison of all mutant discs harboring a mutation to all those that do not harbor it. P values indicated above panels were obtained from comparisons of the two-choice percentage using paired Student's *t*-tests. Solid and dashed lines represent median and quartile values, respectively. Dashed lines represent quartiles.

Discussion

Chemical defenses are an extremely large group of plant protectants. A majority of these metabolites are assumed to be related to protection against pathogens and herbivores (44). However, the effects of very few of these metabolites on insect interactions have been examined in detail using mutant lines. Furthermore, since each plant produces many specialized metabolites and other defenses simultaneously, these compounds may have an altered effect in the context of the entire plant's chemotype.

For this reason, we chose three prominent N. benthamiana defensive compounds and examined their effects, both separately and combined. We found that, as might be expected, different defenses affected different interactors to varying extents. However, acylsugars stood out in almost all of the interaction types. Whether it was flea beetles, aphids, flies, or spiders, these sticky compounds protected plants from the initial visitation stage to the final colonization stage. Since the comparisons we performed were on plants that had reduced abundance of the compounds, instances where only the double *a622+asat2* mutants were susceptible indicate that each compound on its own is enough to protect the plant. In this case, we might ask what advantage *N. benthamiana* gains from producing extremely high amounts of nicotine, which may reach up to 0.4% of leaf dry weight (45), which is similar to ~0.02%-0.04% of leaf fresh weight reported for *N. attenuata*(7, 46). One possible answer is that nicotine, like many other specialized metabolites (47, 48), has a developmental role, in addition to its insecticidal properties (7, 18, 49). This is in line with the smaller sized of our *N. benthamiana a622* mutants and similar recent observation with *N. tabacum* mutants (50). A more likely explanation is that nicotine in *N. benthamiana* serves as a "second line of defense". Almost every potent specialized metabolite that evolved in plants has driven the evolution and adaptation of specialist herbivores, which are more tolerant or altogether resistant to the metabolite. Furthermore, even when not looking at specialists, there are entire classes of herbivores that could just be less susceptible to certain chemical defenses, as was seen with the snails in this study and was reported for slug resistance to alkaloids (51–56).

Plants lacking nicotine, acylsugars, and the serine protease inhibitor are nevertheless lethal to *S. exigua* neonates, demonstrating that they must have additional potent protections. When analyzing the specialized metabolites present in *Nicotiana* species, Drapal *et al.* showed

that a laboratory strain of *N. benthamiana* produced several pyridine alkaloids, chloragenic acids, sterols, terpenes, hydrocarbons, and other specialized metabolites (57). This wide array of chemicals is consistent with the observation that combinations of compounds are typically involved in protecting plants against any given herbivore (58). In contrast to *S. exigua*, snails, which caused immense damage in the field, consumed wildtype plants as readily as the mutant plants. We can therefore assume that, even if we didn't see an effect of nicotine or SerPIN-II there are contexts in which these play a primary role in plant protection. Finally, it is possible that the combination of compounds is greater than the sum of their individual effects. This was elegantly demonstrated by Steppuhn et al. (11), who showed that herbivores could compensate for a protease inhibitor by increasing the amount of plant material they consumed. However, nicotine prevented this compensation response. Such an effect can be seen in the caterpillar growth assays. Nicotine alone, acylsugars alone, and even the combination of the two does not affect tobacco budworm growth. However, adding the *serp2* mutations, which alone was not found to affect insect interactions in any of our assays, results in plants that are more susceptible to *C. virescens*.

In this study, we adopted an approach of hypothesis-based experimentation in a natural setting (59). Using this approach, we recorded every interaction we were able to score, including flies that were resting on leaves for mere seconds, parasitized aphids, and bumblebees pollinating the *N. benthamiana* flowers. This type of experimentation is common practice in many ecological circles but is not often used when more "molecular" trials that include the generation of mutant populations are performed. An approach using mutant plants has the advantage of allowing us to determine the defensive functions of the metabolite combinations, while still leaving room for more complex answers beyond rejecting null hypothesis.

Materials and Methods

For more detailed materials and methods see the supplementary materials.

Plant material and growth conditions

Nicotiana benthamiana plants were grown in temperature-controlled growth chambers or greenhouses prior to field planting and controlled experiments. Plants used for genome editing either expressed the *PCO-CAS9* gene (60), or the same *CAS9* while also harboring a mutation in the *SerPIN-II* gene (43).

Field-grown plants were planted during the summer of 2023 in a deer-excluded field in Ithaca, NY (42.46 °N, 76.44 °W), in 24 blocks of 14, each including two independent isolates of

each mutant line (Fig. S1). Distances between individual plants were 40 cm, with 110 cm between blocks.

Insects and growth conditions

Chloridea virescens and *S. exigua* eggs were purchased from Benzon Research (Carlisle, PA, USA). Two strains of *M. persicae* were used in this study: a green strain nicotine-sensitive strain and a red nicotine adapted strain (61, 62).

Mutant accessions and generation of the mutant population

The mutant population was composed of wildtype plants, mutants of three genes, and their combinations: *SerPIN-II (43)*, *ACYLSUGAR ACYL TRANSFERASE2* (*ASAT2*;(29)), and the *N. benthamiana A622* gene, which was identified by a BLAST search of the *N. benthamiana* transcriptome with known *Nicotiana A622* (41). Three crRNA targets were identified for *A622* and were inserted into a TRV plasmid vector, either on their own or in combination with two crRNAs targeting *ASAT2* (29). The two guide RNA combinations were expressed on two backgrounds, either *CAS9*-expressing plants (63) or *CAS9*-expressing *SerPIN-II* mutants (43).

Genome editing of N. benthamiana plants was performed using TRV expressing guide RNAs as described by Ellison et al. (63), and progeny were grown for analysis by liquid chromatography-mass spectrometry (LC-MS).

Identification and relative quantification of nicotine and acylsugars

Nicotine and acylsugars were identified and relatively quantified using LC-MS, on an Ultimate 3000 HPLC (Thermofisher Scientific) coupled to a Q Exactive quadrupole - orbitrap mass spectrometer (Thermofisher Scientific) using a C18 column. Acylsugars were analyzed similar to Feng et al. (29). Nicotine was analyzed on the same LC-MS system as the acylsugars and was identified using a nicotine standard (Sigma Aldrich; St. Louis, MO, USA), exact mass, and fragmentation patterns.

Field observations

Interactions with invertebrates were observed starting four days after planting. For identification, interactors were photographed or were sampled and further examined under a stereo microscope. All 336 plants were observed during the first two cycles (4-7 d and 18-23 d post-planting), after which blocks with uneven plant death were excluded (on 31-34 d, 45 d and 59 d post-planting). Plant height was recorded at 24, 36-38, 52, and 66 days post-planting, whereas survival was monitored continually. In addition, field-grown plants were sampled for nicotine and acylsugars. This process was similar to that performed in the lab.

Insect assays under controlled conditions

Spodoptera exigua neonates were placed in organza mesh bags on individual *N. benthamiana* leaves. After 11 days, survival was scored, and live caterpillars were weighed. Four-day-old *C. virescens* larvae were weighed, and those weighing 5-12 mg were placed in organza mesh bags on individual *N. benthamiana* leaves, with a single caterpillar in each bag. After four days, the caterpillars were weighed, and survival was assessed. In aphid reproduction assays, five adults were caged on plants. After 24 h adult aphids were removed and only three newly born nymphs were left in each cage. After either eight or nine days, aphids were counted. For choice assays, two leaf discs were placed in 100x15 mm Petri dishes, to which 1.1 ml water was added. These were sealed and 24 h later the numbers of aphids on each of the leaf discs were counted.

Statistical analysis.

Statistical analyses were performed using JMP 16 software (SAS Institute, Cary, NC, USA), using a Chi-square test with a Benjamini-Hochberg correction for non-continuous data, Tukey's HSD test, Student's *t*-tests with Benjamini-Hochberg correction, and paired Student's *t*-tests.

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