Induced responses contribute to rapid plant adaptation to herbivory

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Abstract

Herbivory-induced responses in plants are a typical example of phenotypic plasticity and are thought to be important for adaptation to herbivory. However, direct evidence demonstrating that induced responses increase the multigenerational fitness of plants remains scarce. Here, we experimentally evolved populations of an aquatic plant and its native herbivore for more than 30 generations outdoors. We found that herbivory rapidly increased plant resistance via altering the genotype frequencies, herbivory-induced phenotypic plasticity, and plant microbiota. Together, this study provides direct evidence for the adaptive role of herbivory-induced responses in plants.

Introduction

Plants' ability to change their phenotype under stress conditions is predicted to be beneficial (*1–3*). In response to attacks from herbivores, which can drive rapid ecological and evolutionary changes in plant populations (*4*, *5*), plants often increase the biosynthesis of specialized metabolites that deter herbivores from feeding, reduce insect performance, or attract herbivores' natural enemies (*6–9*). At the same time, herbivory can also alter the microbial communities associated with plants which, in turn, can directly or indirectly affect plant growth and resistance against herbivory (*10–12*). However, herbivory-induced defenses may often have trade-offs with growth and reproduction (*13*, *14*), it remains unclear to which extent herbivory-induced responses contribute to rapid adaptive evolution to herbivory, as multigenerational fitness consequences of the induced responses in the natural habitat of plants are rarely measured.

To address this challenge, we used the hydrophyte *Spirodela polyrhiza* (Araceae, Lemnoideae), and its natural herbivore *Limnaea stagnalis* (freshwater snails) as a model system. *Spirodela polyrhiza* predominately reproduces via clonal propagation, with a generation time of ~5 days outdoors during the growing season. It has a small size, a small genome (*15*), and a very low genetic diversity and mutation rate (*16*), allowing real-time tracking of evolutionary processes through multigenerational experimental evolution outdoors. Here, we first asked whether herbivory drives rapid adaptive evolution in plant populations. Then we asked whether phenotypic plasticity and microbiota contributed to the plants' adaptive evolution.

Results

Snail herbivory drives rapid adaptation in S. polyrhiza *outdoors*

We experimentally evolved replicated populations of a mixture of four genotypes of *S. polyrhiza* (Sp21, Sp56, Sp58, and Sp65; Tab. S1) under multigenerational herbivory by the snail *L. stagnalis* or control conditions, respectively. These four genotypes were used since they represent the genetic variation typically found in natural populations in Europe (*16*). We replicated experimental populations over ten ponds outdoors, each hosting two cages with fine nets separating the two treatments (with or without herbivores) while maintaining plants in a common water body (Fig. 1 and Fig. S1). While tracking changes in plant morphology over the course of two growing seasons (Fig. 1a and 1b), we performed a series of experiments to test whether multigenerational herbivory can drive rapid adaptive evolution in plant populations.

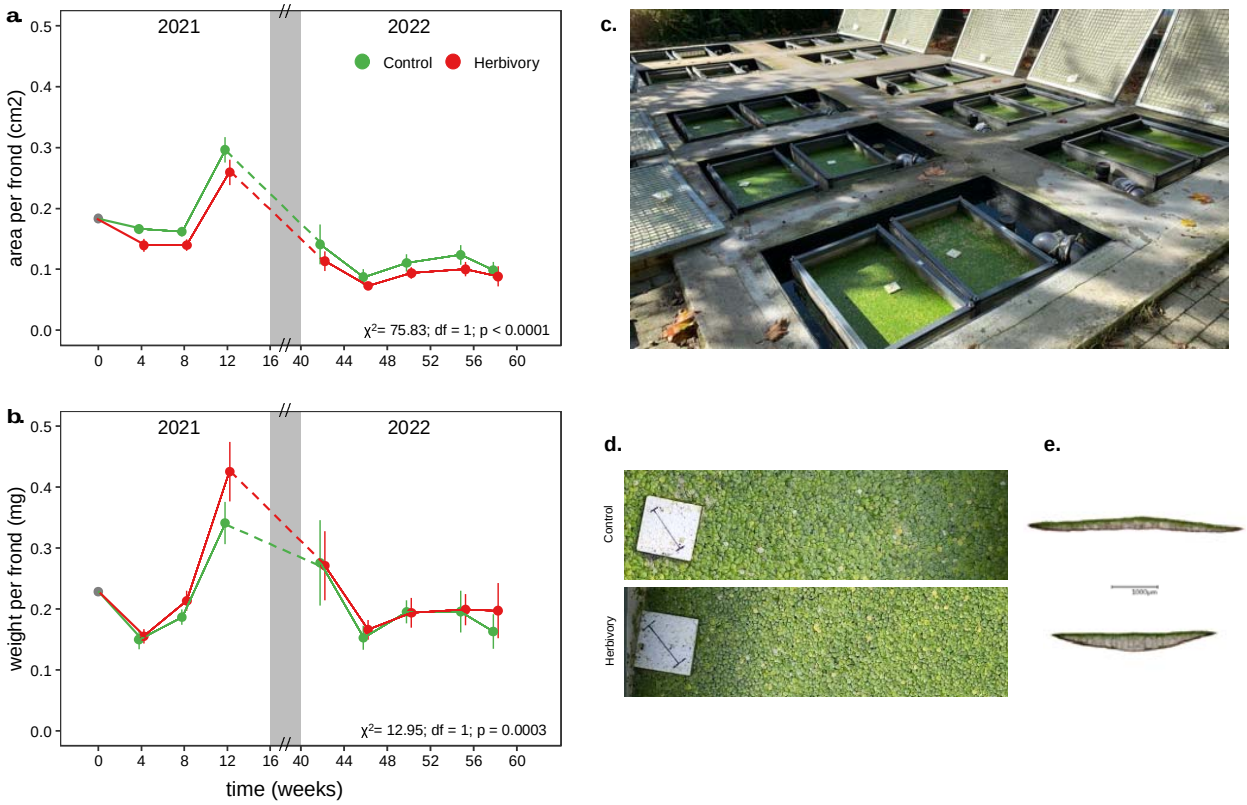

Figure 1. Outdoors experimental setup. Surface area (a) and dry biomass (b) of 500 *Spirodela polyrhiza* fronds exposed to multigenerational herbivory across two growing seasons (2021 and 2022). For each time point, dots represent mean values and bars show the standard deviation. Grey area represents winter. Each plot reports the results from fitting a linear mixed-effects model using "treatment" (herbivory/control) as fixed effect, and "time" and "pond" as random factors. Plant populations that experienced multigenerational herbivory outdoors showed a lower surface area and a higher dry weight per frond compared to the control group (Tab. S2). Overview (c) of the experimental setup with the 10 ponds each hosting two cages. Differences in frond morphology from top (d) and cross sections (d) in fronds grown in control cages (upper) or herbivory cages (lower). In both treatment groups, the surface of each cage was fully covered by *S. polyrhiza* within 2 weeks, and none of the populations collapsed due to herbivory or other reasons within the first growing season (2021), while three populations collapsed during the second growing season (2022).

To test the effect of evolution under multigenerational herbivory on plant growth rate, we randomly selected four groups of 50 individual plants (also called fronds) from both control and herbivory treatments from each pond at week 8. Fronds were isolated from herbivores while keeping them in the same cage, and we measured their growth (changes in frond number, biomass, surface area) during a 2-weeks period. The results showed that populations of *S. polyrhiza* that had previously been exposed to herbivory grew more slowly in terms of number of fronds (χ^2= 36.72; df = 1; p < 0.0001; Fig. S2a), biomass (χ^2= 706.37; df = 1; p < 0.0001; Fig. S2b), and area (χ^2= 26794; df = 1; p < 0.0001; Fig. S2c) in comparison to populations that did not experience herbivory. Cross sections

on typical fronds revealed that multigenerational herbivory influenced plant morphology (Fig. 1). Together, these results show that *S. polyrhiza* populations grew smaller, slower, and heavier under multigenerational snail herbivory.

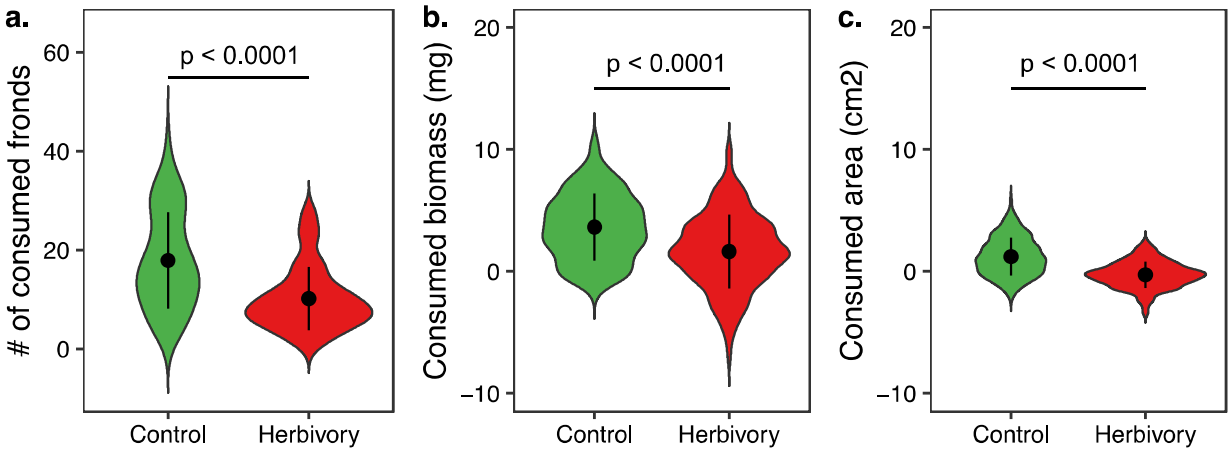

Figure 2. **Consumed number of fronds (a), biomass (b), and surface area (c) of *Spirodela polyrhiza* fronds that did or did not experience multigenerational herbivory**. Values for plots (b) and (c) are calculated by permutation (*n* = 999) with a set of reference fronds collected before the bioassay. For each group, dots represent mean values and bars show the standard deviation.

To test if the phenotypic plasticity we observed was adaptive, we compared plant resistance to the snails (during 24 h) between plants grown under multigenerational herbivory or control conditions. We found that snails consumed fewer fronds (χ^2= 35.74; df = 1; p < 0.0001; Fig. 2a), less biomass (χ^2= 449.61; df = 1; p < 0.0001; Fig. 2b), and less surface area of fronds (χ^2= 730.22; df = 1; p < 0.0001; Fig. 2c) from the populations that had previously experienced multigenerational herbivory than the control populations. In addition, we also found that herbivory altered the production of putative defensive plant metabolites during two consecutive growing seasons (Fig. S3), inducing a consistent increased production of tyramine. Taken together, these results suggest that multigenerational herbivory reduced the growth rate, altered plant morphology and metabolism, and increased herbivore resistance in *S. polyrhiza* populations.

Phenotypic plasticity and evolution jointly contributed to the rapid adaptation

To investigate whether the observed rapid phenotypic changes are due to changes in genotype frequency or phenotypic plasticity, we first quantified frequencies of each genotype using a pool-seq approach on samples collected after 8 and 12 weeks of experimental evolution. We found that genotype frequency was influenced by treatment (Tab. S3). In comparison to the starting population (25% of each genotype), herbivory significantly altered the genotype frequencies of Sp21 and Sp65

in opposite directions (Fig. 3), while it did not influence the frequency of genotypes Sp56 and Sp58. At week 12, in comparison to controls, while herbivory reduced the frequency of genotype Sp21 (control 33.9±2.4%, herbivory 24.5±1.8%, p = 0.0004), it increased the frequency of genotype Sp65 (control 19.3±1.6%, herbivory 27.1±2.1%, p = 0.0006), suggesting that herbivory rapidly altered genotype frequencies in the duckweed populations outdoors.

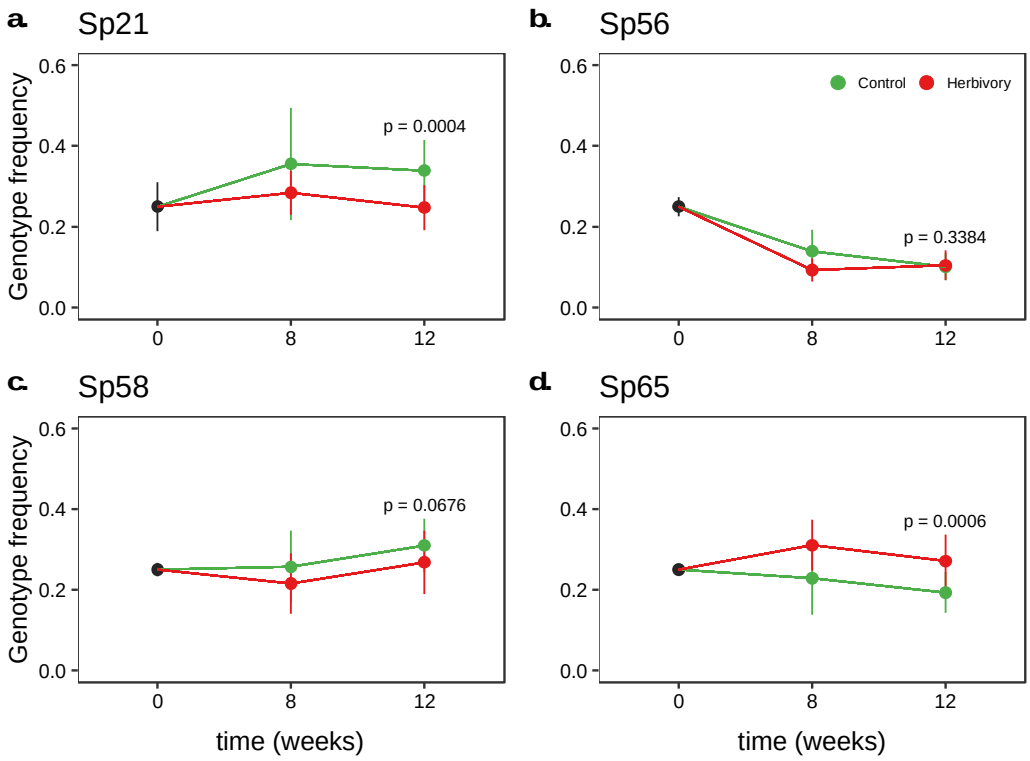

Fig. 3. **Relative frequency of *Spirodela polyrhiza* genotypes Sp21 (a), Sp56 (b), Sp58 (c), and Sp65 (d) at the beginning of the experiment and after experimental evolution under multigenerational herbivory or in control conditions during the first growing season (~ 30 generations at week 12)**. For each group, dots represent mean values and bars refer to the standard deviation.

We then tested the hypothesis that intrinsic differences in growth and resistance among genotypes contributed to the observed adaptive changes. To this end, we grew 30 fronds of each genotype under indoor sterile conditions, using genotypes directly from our collection. We measured their growth rate and resistance to herbivory. While the growth rate differed among genotypes ($F_{3,\ 35}$ = 3.51, p = 0.02; Fig. S4a), post-hoc contrasts did not show pairwise differences between them, suggesting that the overall differences were marginal. Herbivory resistance assays showed that the four genotypes differed in their intrinsic resistance ($F_{3,\ 44}$ = 58.62, p < 0.0001; Fig. S4b), which is mainly due to genotype Sp56 that is more susceptible to herbivory than the others (p < 0.0001). No

differences were found in Sp21 and Sp65, the frequency of which were altered by herbivory, suggesting that neither the intrinsic growth rate nor resistance contributed to the observed rapid changes in resistance.

To further investigate whether herbivory *per se* can contribute to the observed phenotypic changes, we then experimentally subjected each genotype separately to the snail's attack or control conditions, respectively, in microcosms indoors. After eight weeks, we found that the fronds of all genotypes attacked by herbivores were smaller (Fig. S5 and Tab. S4), indicating that phenotypic plasticity can contribute to the observed morphological changes outdoors.

To further disentangle the effects of phenotypic plasticity and changes of genotype frequencies, we then assembled four different artificial populations using a full factorial design. Here, we used individual genotypes grown under control conditions or herbivory, representing absence or presence of induced responses, respectively. Artificial populations were assembled using the genotype frequencies observed in control or herbivory populations outdoors (Fig. 4), thus representing the presence or absence of evolutionary effects. We then quantified the levels of resistance using 24 h herbivory assays. We found clear interaction effects between induced responses and evolutionary changes in the genotype composition (plasticity × evolution χ^2= 4.47; df = 1; p = 0.034; Fig. 4). While the changes in genotype frequency did not alter plant resistance in populations assembled using control plants (p = 0.914; Fig. 4), they increased the resistance to herbivory when plants were previously induced by herbivory (p = 0.006; Fig. 4). Together, the results suggest that both herbivory-induced phenotypic plasticity and evolution contributed to the rapid increase of resistance in *S. polyrhiza* outdoors.

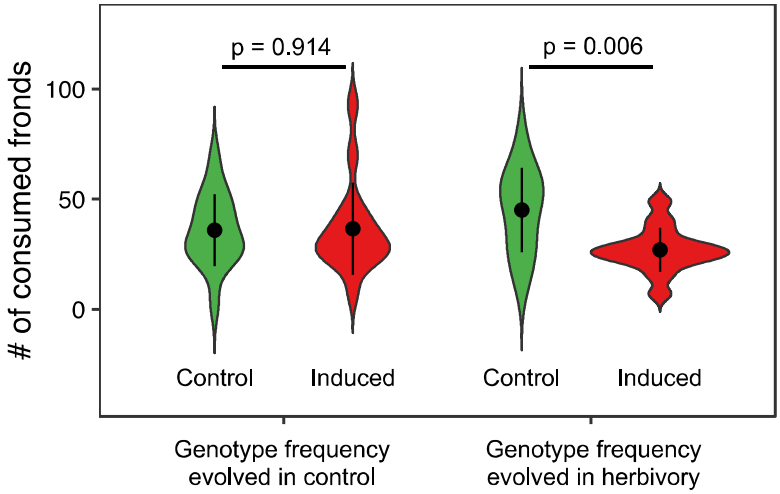

Figure 4. **Number of consumed fronds after 24h of herbivory in synthetic populations**. These populations created by mixing fronds from single genotypes according to the genotype frequencies we observed in the control (Sp21 36%; Sp56 10.7%; Sp58 32.9%, Sp65 20.4%) or herbivory (Sp21

27.8%; Sp56 11.7%; Sp58 30.1%, Sp65 30.4%) cages in the outdoors setup. Genotypes were either grown under multigenerational herbivory (induced, red) or control (non-induced, green) conditions. For each group, dots represent mean values and bars show the standard deviation.

Herbivory-induced microbiota contributed to rapid adaptive evolution to herbivory

As herbivory can also induce changes in plant microbiota, we then examined whether herbivory-induced plant microbiota contributed to the observed adaptive changes outdoors. First, we characterized the duckweed-associated microbiota using the pool-seq data, and we found that herbivory altered the structure of the microbial communities associated with *S. polyrhiza* (Tab. S5). To test whether the changes in microbiota caused changes in plant growth and resistance, we took a natural microbiota inoculation approach by growing each individual genotype within the cage of our experimental setup outdoors. These plants were kept separated from direct herbivory using a fine net, so they were only exposed to the microbiota, either induced by herbivory or control, respectively, in the native environment of the experimental cages. After eight weeks outdoors, we measured the growth rate and resistance to snail herbivory of individual genotypes outdoors. The results show that, while herbivory-induced microbiota did not alter the plant growth measured by either biomass or surface area (Tab. S6), it increased the resistance to snail herbivory, as quantified by 24 h feeding assays, in genotype Sp65 ($\chi^2 = 3.78$; df = 1; $p = 0.05$; Fig. 5a). No differences were found in the other three genotypes ($p > 0.05$; Fig. 5a; Tab. S7). Because herbivory-induced microbiota had different effects on the resistance level in Sp21 and Sp65, we then characterized the plant-associated microbial community of genotypes Sp21 (Fig. 5b) and Sp65 (Fig. 5c), which genotype frequency was altered by herbivory, using an amplicon metagenomics approach. While herbivory induced little changes in the microbiota associated with Sp21 ($F_{1, 12} = 1.40$; $p = 0.06$; Fig. 5b; Tab. S8), herbivory significantly altered the bacterial microbiota of Sp65 ($F_{1, 12} = 1.58$; $p = 0.01$; Fig. 5c; Tab. S8), consistent with the observed differences in resistance between the two genotypes. No differences between the two treatments were found when investigating the structure of the eukaryotic community associated with genotypes Sp21 ($F_{1, 12} = 0.73$; $p = 0.29$) and Sp65 ($F_{1, 12} = 0.52$; $p = 0.42$). These results suggest that herbivory-induced changes in plant microbiota contributed to the rapid adaptive evolution of *S. polyrhiza* outdoors.

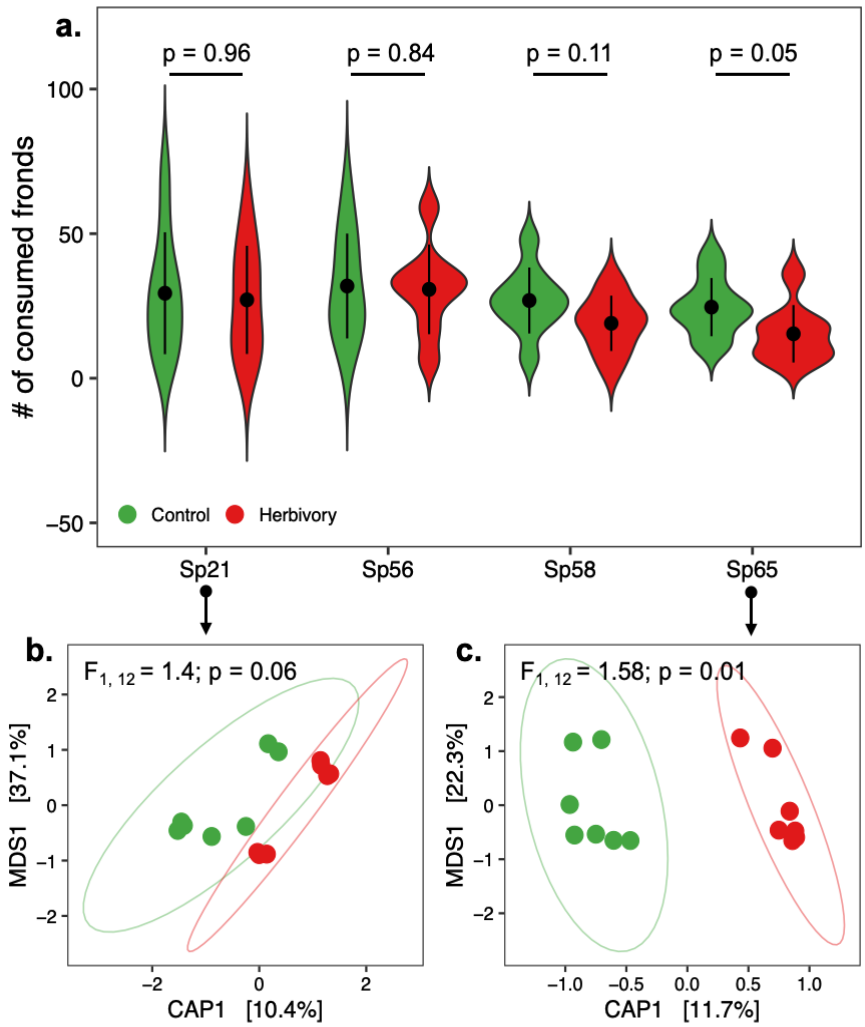

Figure 5. **Resistance to herbivory and changes in the microbial community of individual *Spirodela polyrhiza* genotypes**. (a) Number of consumed fronds after 24h of herbivory for each *S. polyrhiza* genotype, grown for 8 weeks in cages with (red) or without (green) herbivores, but protected from snails using fine nets. For each group, dots represent mean values and bars show the standard deviation. (b) and (c) Canonical analysis of principal coordinates (CAP) analysis and PERMANOVA of bacterial communities (Unifrac distance matrix) for genotype Sp21 (b) and genotype Sp65 (c).

Discussion

Here, by carrying out multigenerational experimental evolution outdoors, we demonstrated that herbivory-induced responses contributed to herbivory-driven multigenerational adaptive evolution in *S. polyrhiza*. We found that herbivory reduced plant size and growth rate but increased resistance to herbivory. Morphological changes in the population were found already after four weeks of herbivory and were observable after more than 30 asexual generations, indicating the observed phenotypic

changes are robust to abiotic environmental changes. These changes are likely caused by direct herbivore feeding but not microbiota, as plants growing in the same microbiota did not show clear morphological changes. Although the four genotypes differ in their size, the observed morphological changes in the populations are likely due to induced responses (phenotypic plasticity) but not the changes in genotype frequencies. This is because the size differences were mostly found between Sp56 and the other three genotypes, but the frequency of Sp56 was not altered by herbivory. In addition, when individual genotypes were attacked by snail herbivory, similar morphological changes were found in all four genotypes.

The observed rapid increases in resistance to the snail herbivory were likely due to the combination of herbivory-induced changes in phenotypic plasticity, evolution, and microbiota. For instance, sole changes in genotype frequency or induced responses did not alter the resistance to herbivory (Fig. 4). This is likely because herbivory induced different levels of resistance among genotypes, and the selection imposed by herbivory acted on the induced resistance traits. Using a targeted metabolomics approach, we observed changes in several candidate defensive metabolites, which were induced by multigenerational herbivory across two growing seasons. However, it is also possible that the increased resistance might be due to thickened cell-walls, or unknown defensive metabolites that altered the feeding choice of the herbivores.

We found that the herbivory-induced changes in bacterial community contributed to the increased resistance in Sp65 (Fig. 5), the frequency of which was increased in the outdoors experiment. Interestingly, the changes in microbiota were not found in other genotypes, indicating the herbivory-induced changes in microbiota are genotype-specific, which can be directly subject to natural selection. Although several microbial taxa were found specifically altered by herbivory in Sp65, it remains unclear which specific bacteria caused the observed changes in resistance.

Together, this study provides direct evidence supporting the long-standing hypothesis that herbivory-induced responses are adaptive (*17*). The potential adaptive role of herbivory-induced microbiota further highlighted the importance of conducting long-term outdoor experiments for understanding how plants respond to environmental stresses.

Methods

Experimental evolution

This experiment was set up during 2021 in Münster (Germany) using *Spirodela polyrhiza* as model species and included 10 experimental ponds (120 × 150 × 100 cm). About seven weeks before the beginning of the experiment, ponds were cleared from any residual from the previous year. We also added 40 L of commercial potting soil (Floragard) and 550 mL of commercial liquid fertilizer (Compo BIO) to each pond to support plant growth throughout the season. A few days before beginning the

experiment we homogenized the water among all the ponds. Each pond was then divided in two compartments using two cages (105 × 74 × 44 cm) with sides covered with a fine metal net (0.14 mm). Each pond was covered with a metal grid lined with a shading net, ensuring optimal growth conditions, and avoiding large debris and small animals to fall inside the pond.

Before experimental setup, we separately grew four genotypes of *S. polyrhiza* (Sp21, Sp56, Sp58, Sp65, Tab. S1) in controlled conditions (26°C, 16h:8h, 135 µmol/m light) for ~4 weeks in full N-Medium (KH_2PO_4 150 µM, $Ca(NO_3)_2$ 1 mM, KNO_3 8 mM, H_3BO_3 5 µM, $MnCl_2$ 13 µM, Na_2MoO_4 0.4 µM, $MgSO_4$, 1 mM, FeNaEDTA 25 µM) (*18*), and we moved them for ~1 week to a semi-controlled greenhouse, helping their transition to outdoors conditions.

At the starting day of experimental evolution (June 9th, 2021), for each cage we added 500 fronds of each *S. polyrhiza* genotype. In addition, within each cage we added 4 floating boxes (11.5 × 11.5 × 7.5 cm) with fine metal net on the bottom, and each box received 150 fronds of a single genotype. In this way, mixed fronds within each cage were used for experimental evolution, while fronds of single genotypes within floating boxes were used to test the indirect effect of each treatment (control/herbivory, see below) on single genotypes.

Each pond served as a paired unit to compare the effect of herbivory on the evolution of aquatic plant populations. Keeping the paired design in mind, we randomly assigned each cage to a control (n = 10) or herbivory treatment (n = 10). Cages assigned to the herbivory treatment were inoculated with 12 snails (*Lymnaea stagnalis*, 18-22 mm of shell length) over a period of 3 weeks, to ensure that they would not lead to the collapse of the entire plant population. At the experimental setup, we stored 150 fronds of each genotype (in triplicate) to be used as reference to estimate genotype frequencies (see below). We also measured surface area and dry weight of 30 fronds from each genotype. In addition, at experimental setup, we created an artificial mix by using 500 fronds of each genotype, mixing them into a bucket, and sampling 400 fronds (in triplicate). This artificial mix was processed for pool-seq, and data was used to normalize genotype frequencies in an attempt to reduce sampling bias (see below).

After 4, 8, and 12 weeks from the experimental setup, from each cage we collected two sets of 500 fronds. Each set was used to measure frond surface area, then it was freeze-dried to measure dry weight, and finally processed either for pool-seq or for metabolomics (see below). In addition, after 4 and 8 weeks from experimental setup, we collected 30 fronds from each genotype grown in the floating boxes, which were used to measure surface area and dry weight. Surface area was measured for each sample by taking a picture of fronds right after sampling and processing it with the R package pliman (*19*) in R v.4.1.2 (*20*). Dry weight was measured after freeze-drying samples.

Data was analyzed using R v.4.1.2 (*20*) with the packages *lme4* v1.1.29 (*21*) and *car* v3.0.13 (*22*), using different strategies as specified for each test below. Pairwise contrasts were performed using

the package *emmeans* v.1.7.4 (*23*), correcting p-values using the False Discovery Rate (FDR) method.

The overall effect of herbivory on the mixed population outdoors over 2021 and 2022 was tested by fitting two linear-mixed effect models, one using frond surface area and the second using dry weight as variable and specifying *treatment* (herbivory/control) as fixed factor, and *pond* and *timepoint* as random factors, using the formula: ~ treatment * (1|week) * (1|pond).

Growth and herbivory assays outdoors

Growth and herbivory assays were designed to test the influence of evolution under herbivory on plant growth (change in number of fronds, biomass, and surface area) or resistance to herbivory (number of consumed fronds, consumed biomass, and consumed surface area). Growth assays on the mixed population were carried out on the 9th week of experimental evolution. For this assay, we used the same floating boxes used to grow the individual genotypes (see below), after being cleared from any frond. Within each cage, we randomly picked fronds from 5 different spots, mixed them thoroughly, and randomly selected 50 fronds to be added to each floating box (n = 4 boxes for each cage, n = 80 in total). Then, after 10 days of growth, we collected all the fronds, counted them, took a picture to estimate the surface area, and freeze dried them to measure the dry weight.

Relative changes in frond biomass and area were estimated by permutation (n = 999) using the data from the mixed population monitoring (see above) before the beginning of this bioassay. The effect of herbivory on plant growth was tested by fitting a linear mixed-effects model specifying *treatment* (herbivory/control) as fixed factor, and *pond* as random effect, using the formula: ~ treatment * (1|pond).

Herbivory assays were carried out for both single genotype (week 8) and mixed populations (week 10). Assays using single genotypes were performed right before the growth assay described above. For each floating box, we reduced the number of fronds to 100 and we added a single snail that has been starved for 24 h before the assay. After 24 h of herbivory, we removed all the snails, collected all the fronds, counted them, took a picture to estimate the surface area, and freeze dried them to measure the dry weight. Resistance was estimated as number of consumed fronds, and the effect of herbivory on resistance was tested by fitting a linear mixed-effects model specifying *treatment* (herbivory/control) and *genotype* as fixed factors, and *pond* as random effect, using the formula: ~ treatment * genotype * (1|pond).

Herbivory assays using the experimentally evolved populations were performed in a similar way after the growth assay described above (week 10). Floating boxes (n = 4 boxes for each cage) were cleared, and each received 50 fronds randomly picked from the population within the same cage. Then we added a single snail as described above, allowing it to feed for 24 h. After removing all the snails, we collected all the fronds, counted them, took a picture to estimate the surface area, and

freeze dried to measure the dry weight. The entire procedure was repeated over two consecutive rounds, separated by 1 day to allow the starvation of the snails resulting in n = 40 per treatment (herbivory vs control). The effect of multigenerational herbivory on resistance was tested by fitting a linear mixed-effects model specifying *treatment* (herbivory/control) as fixed factor, and *pond* and *bioassay round* as random effects, using the formula: ~ treatment * (1|round) * (1|pond).

Pool-seq

We used pool-seq with the double goal of testing changes in genotype frequency and plant-associated microbial community as a consequence of multigenerational herbivory. Samples included a group of 400 fronds randomly picked from each cage at week 8 and 12 (n = 20 for each week). In addition, we included three sets of samples to be used as a reference: (i) a set of three replicates for each plant genotype used to begin the evolution experiment (n = 12); (ii) a set of three replicates where we mixed equal number of fronds of each genotype (n = 3); (iii) a set of three replicates obtained by mixing 500 fronds of each genotype, mixing them thoroughly, and sampling 400 fronds (n = 3). These three sets of reference samples were then used to reduce the bias of the genotype frequency data (see below). DNA was extracted from ~10 mg of plant material from each sample using a phenol:chloroform protocol, and then shipped to Novogene Ltd. for shotgun metagenomics library preparation and sequencing on a Illumina NovaSeq 6000 S4 150PE flow cell.

Raw data was processed using TrimGalore v0.6.7 (https://github.com/FelixKrueger/TrimGalore) to remove Illumina adaptors and discard low-quality reads. Then, we used Bowtie2 v2.4.5 (*24*), samtools v1.14 (*25*) and the *S. polyrhiza* reference genome (*15*) to map the raw data and split reads for each sample to those matching the reference genome (to be used to estimate genotype frequency) and those not matching the reference (to be used to infer the structure of the plant microbiome).

Genotype frequency from pool-seq

For each sample, the BAM files of reads matching the *S. polyrhiza* reference genome were processed to estimate the frequency of each genotype using HAFpipe (*26*) together with the reference genome (*15*), a VCF file with all the variable sites between the 4 genotypes (*16*) and setting the recombination rate to 0 (we also tried setting it to 0.00051 as in Ho et al. (*27*), and results did not change), the number of generations to 30, and the window size to 100bp. Then, we used the data from the reference fronds for each individual genotype to identify loci that: (i) identify a given genotype with a confidence > 90 %, (ii) are common to all four genotypes. This allowed us to identify 68 loci that we used to estimate the frequency of the four genotypes for each sample. First, we tested whether this approach correctly identifies our reference samples from individual genotypes, and we found that the misassignment error was always <1.9 %, which is low. Then, we tested this

pipeline on the samples where we mixed fronds from each genotype in equal numbers, and we found that the relative abundance of individual genotypes was different from 25 % each. We expected this result, as fronds from different genotypes have different size and biomass (see results), and this might affect the amount of DNA extracted from each of them. Given that also the sampling itself might add an error in the frequency estimates, we used the reference samples where we mixed an equal number of fronds from each genotype and then mixed them before subsampling to correct the genotype frequencies for experimental samples. Then, using only data from experimental samples at week 8 and 12, we fit a linear mixed-effects model (~ treatment * genotype * (1|timepoint) * (1|pond)) and extracted the pairwise comparisons between treatments (herbivory/control) for each genotype using *emmeans*.

Metagenomics analyses from pool-seq

Using samtools, we converted the BAM files of data not matching the reference genome of *S. polyrhiza* into FASTQ files, which were then used as input for Kraken2 v2.1.2 (*28*) to characterize the composition of the plant-associated microbial communities. This analysis was done twice, first using the Kraken2 reference database for archaea, bacteria, and fungi, and then using a custom database for algae built using all RefSeq genomes from NCBI for this group (Accessed on March 1st, 2022). The output from Kraken2 was then used to estimate the abundance for each taxon using Bracken v2.7 (*29*). Data was then collated into an abundance matrix, all singletons we discarded, and data was normalized using DESeq2 (*30*). Shannon's diversity index was estimated for each sample using the package *microbiome* (https://microbiome.github.io/), and we tested the influence of treatment and sampling timepoint by fitting a linear mixed-effects model (~ treatment * timepoint * (1|pond)). We also tested the effect of treatment and sampling timepoint on the structure of microbial communities using a PERMANOVA on a Bray-Curtis distance matrix between samples (999 permutations, stratified at the "pond" level), and visualizing this multivariate dataset using a NMDS approach. Taxa that were differentially abundant between treatments (herbivory/control) were identified for each timepoint (week 8 and week 12) using DESeq2 with a FDR-corrected $p<0.05$.

Water analyses

Nutrient levels for each pond water were analyzed using ion chromatography (792 basic IC, 732 detector, Metrohm, Herisau, Switzerland). Samples were taken every two weeks (from experimental setup to week 12) during the first season (n=10 per treatment cage) and once during the second growth season at week 58 (n=7 per treatment cage). We sampled 10 mL per cage for anion and cation analysis. When collecting samples from each cage, we pooled water from five different locations at a depth of 10 cm below the water surface. Anion samples were measured right away, while pH of cation samples was adjusted to pH 3 using 25 % HNO_3 before storage at -20°C until

analysis. Anions (Cl, NO_2, NO_3, PO_4, SO_4) were measured using: column Metrosep A Supp 5 150, 4.0 x 150 mm, eluent 3.2 mM Na_2CO_3 and 1 mM $NaHCO_3$, flow rate 0.7 ml/min, injection volume 20 µl. Cations (Na, NH_4, K, Ca, Na) were measured using: column Metrosep C2 250, 4.0 x 250 mm, eluent 1.7 mmol/L nitric acid, 0.7 mmol/L dipicolinic acid, flow rate 0.9 ml/min, injection volume 20 µl. Quantification was based on external standard ion mixture using a 6-point-reference curve (0.5, 5, 10, 25, 50, 100 mg/L for anions and 0.5, 5, 10, 15, 25, 50 m/L for cations). Throughout the growth seasons we measured the pH of each pond using a Cond3310, (WTW, Xylem Analytics) instrument. The effect of herbivory on water nutrients and pH was tested by fitting a linear mixed effects model specifying *treatment* (herbivory/control) as fixed factor, and *pond* and *timepoint* as random effects, using the formula: ~ treatment * (1|timepoint) * (1|pond). In addition, we tested the effects of overall nutrient content through PERMANOVA on data collected at week 4, 6, 8, 10, and 12 (999 permutations, stratified over "pond" and "sampling timepoint"). Results show that the nutrient levels (Tab. S9, Fig. S6) and pH (Tab. S9) were similar in both cages within each pond throughout the first season.

Metabolomics

To test changes in the production of a wide set of metabolites as effect of multigenerational herbivory, we collected a similar set of 400 fronds randomly picked from each cage at week 12 (season 1) and week 58 (season 2) (n = 20 cages for each week), contextually to the sampling for pool-seq described above. The list of metabolites and the detailed experimental procedures are described more in detail in the supplementary text below. We tested the effect of multigenerational herbivory (herbivory/control) on each metabolite separately for week 12 (season 1) and week 58 (season 2) by fitting a linear mixed effect model for each metabolite using the formula ~ treatment * (1|pond).

Amplicon metagenomics

We tested whether genotypes Sp21 and Sp65 grown outdoors under the indirect effects of herbivory or control conditions associated with a different microbial community. We selected a group of 30 fronds from each genotype from each cage (n = 40), and extracted DNA as indicated above. Samples were then shipped to Novogene Ltd. for amplicon metagenomics library preparation and sequencing on an Illumina NovaSeq 6000 SP 250PE flow cell. Libraries targeting the bacterial community were built amplifying the V4 region of the 16S rRNA (primers 515F and 806R), while those targeting the eukaryotic community (including algae and fungi) were built amplifying the V4 region of the 18S rRNA (primers 528F and 706R).

Raw data was processed using TrimGalore v0.6.7 to remove Illumina adaptors and discard low-quality reads. Paired-end reads were processed using the DADA2 v1.22 (*31*) pipeline implemented

in R v4.1.2 (*20*) to remove low-quality data, identify ASVs and remove chimeras. Taxonomy was assigned using the SILVA v138 database (*32*) for 16S data, or the PR2 v4.14 database (*33*) for 18S data. Data was then analyzed using R v4.1.2 with the packages phyloseq v1.38 (*34*), vegan v2.6 (*35*), DESeq2 1.34 (*30*), and lme4 1.1.29 (*21*). Distances between pairs of samples, in terms of community composition, were calculated using an unweighted Unifrac matrix, then visualized using a CAP (canonical analysis of principal coordinates) procedure. Differences between sample groups were inferred through permutational multivariate analysis of variance (PERMANOVA, 999 permutations) separately for each genotype, specifying treatment (herbivory/control) as fixed factor, and using the factor "pond" to stratify permutations. Taxa that were differentially abundant between treatments (herbivory/control) were identified for each genotype using DESeq2 with a FDR-corrected $p<0.05$.

Experimental evolution for single genotypes indoors

With the goal of enhancing the support to our results obtained outdoors, we replicated our experimental evolution setup indoors (greenhouse, kept at 23°C throughout the experiment). In this case, we exposed each genotype of *S. polyrhiza* to multigenerational herbivory (~30 generations) individually. Then, we assembled synthetic plant communities according to the genotype frequencies we observed outdoors at week 12, and we ran both growth and herbivory assays to disentangle the effect of adaptation to herbivory and frequency of each genotype in influencing the resistance and growth within the plant population.

Each genotype was grown individually in two floating boxes (11 × 11 × 8 cm) inside 5 experimental ponds (60 × 40 × 27 cm), with a total of 8 boxes per pond. As indicated above, floating boxes were modified to allow water (and microbes) to freely flow between the box and the pond, without allowing snails to leave the box. At the experimental setup, we used water from the ponds outdoors, and for each floating box we added 600 of each *S. polyrhiza* genotype accordingly. For each genotype, one group was exposed to herbivory by a single snail (*L. stagnalis*) placed inside the floating box, while the other was used as a control.

After 8 weeks of experimental evolution, we performed a growth assay within the same ponds. The plant population within each floating box was reduced to 200 and herbivores were removed. After 14 days, we counted all the fronds and collected a sample of 50 fronds from each box to measure surface area and dry weight. Right after the growth assay, we performed an herbivory assay using four synthetic populations, two assembled using the genotype frequencies observed outdoors in ponds with herbivores, and two assembled using the genotype frequencies observed in control ponds outdoors. Synthetic populations were assembled either with genotypes that experienced multigenerational herbivory or with plants grown in control conditions. The herbivory assays were

replicated over the 5 ponds, exposing 100 fronds to a single starved snail for 24 h. Then, we counted residual fronds and measured the surface area and dry weight as reported above.

The effect of multigenerational herbivory on resistance was tested by fitting a linear mixed-effects model specifying *population* and *treatment* (herbivory/control) as fixed factors, and *pond* as random effect, using the formula: ~population * treatment * (1|pond), extracting the pairwise comparisons between treatments (herbivory/control) for each genotype using *emmeans*.

References

1. A. D. Bradshaw, Unravelling phenotypic plasticity -- why should we bother? *New Phytol.* **170,** 644–648 (2006).
2. A. D. Bradshaw, "Evolutionary Significance of Phenotypic Plasticity in Plants" in *Advances in Genetics*, E. W. Caspari, J. M. Thoday, Eds. (Academic Press, 1965), vol. 13, pp. 115–155.
3. C. D. Schlichting, The evolution of phenotypic plasticity in plants. *Annu. Rev. Ecol. Syst.* **17**, 667–693 (1986).
4. A. A. Agrawal, A. P. Hastings, M. T. J. Johnson, J. L. Maron, J.-P. Salminen, Insect herbivores drive real-time ecological and evolutionary change in plant populations. *Science*. **338**, 113–116 (2012).
5. T. Züst, C. Heichinger, U. Grossniklaus, R. Harrington, D. J. Kliebenstein, L. A. Turnbull, Natural enemies drive geographic variation in plant defenses. *Science*. **338**, 116–119 (2012).
6. M. Dicke, R. M. P. van Poecke, J. G. de Boer, Inducible indirect defence of plants: from mechanisms to ecological functions. *Basic Appl. Ecol.* **4**, 27–42 (2003).
7. A. Steppuhn, K. Gase, B. Krock, R. Halitschke, I. T. Baldwin, Nicotine's defensive function in nature. *PLoS Biol.* **2**, E217 (2004).
8. M. C. Schuman, K. Barthel, I. T. Baldwin, Herbivory-induced volatiles function as defenses increasing fitness of the native plant *Nicotiana attenuata* in nature. *Elife*. **1**, e00007 (2012).
9. M. E. Fritzsche Hoballah, T. C. J. Turlings, Experimental evidence that plants under caterpillar attack may benefit from attracting parasitoids. *Evol. Ecol. Res.* **3**, 583–593 (2001).
10. J. Friman, P. N. Karssemeijer, J. Haller, K. de Kreek, J. J. A. van Loon, M. Dicke, Shoot and root insect herbivory change the plant rhizosphere microbiome and affects cabbage-insect interactions through plant-soil feedback. *New Phytol.* **232**, 2475–2490 (2021).
11. P. T. Humphrey, N. K. Whiteman, Insect herbivory reshapes a native leaf microbiome. *Nat Ecol Evol*. **4**, 221–229 (2020).
12. L. Hu, C. A. M. Robert, S. Cadot, X. Zhang, M. Ye, B. Li, D. Manzo, N. Chervet, T. Steinger, M. G. A. van der Heijden, K. Schlaeppi, M. Erb, Root exudate metabolites drive plant-soil feedbacks on growth and defense by shaping the rhizosphere microbiota. *Nat. Commun.* **9**, 2738 (2018).
13. I. T. Baldwin, Jasmonate-induced responses are costly but benefit plants under attack in native populations. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 8113–8118 (1998).
14. R. Karban, I. T. Baldwin, *Induced responses to herbivory* (University of Chicago Press, 1997).
15. W. Wang, G. Haberer, H. Gundlach, C. Gläßer, T. Nussbaumer, M. C. Luo, A. Lomsadze, M. Borodovsky, R. A. Kerstetter, J. Shanklin, D. W. Byrant, T. C. Mockler, K. J. Appenroth, J. Grimwood, J. Jenkins, J. Chow, C. Choi, C. Adam, X.-H. Cao, J. Fuchs, I. Schubert, D. Rokhsar, J. Schmutz, T. P. Michael, K. F. X. Mayer, J. Messing, The *Spirodela polyrhiza* genome reveals insights into its neotenous reduction fast growth and aquatic lifestyle. *Nat. Commun.* **5**, 3311 (2014).
16. S. Xu, J. Stapley, S. Gablenz, J. Boyer, K. J. Appenroth, K. S. Sree, J. Gershenzon, A. Widmer, M. Huber, Low genetic variation is associated with low mutation rate in the giant duckweed. *Nat. Commun.* **10**, 1243 (2019).

17. R. Karban, J. H. Myers, Induced plant responses to herbivory. *Annu. Rev. Ecol. Syst.* **20**, 331–348 (1989).
18. K.-J. Appenroth, S. Teller, M. Horn, Photophysiology of turion formation and germination inSpirodela polyrhiza. *Biol. Plant.* **38**, 95 (1996).
19. T. Olivoto, Lights, camera, pliman! An R package for plant image analysis. *Methods Ecol. Evol.* **13**, 789–798 (2022).
20. R Core Team, R: A language and environment for statistical computing (2020).
21. Bates, Mächler, Bolker, Walker, Fitting linear mixed-effects models using lme4. *arXiv preprint arXiv:1406. 5823* (2014).
22. J. Fox, S. Weisberg, *An R companion to applied regression* (SAGE Publications, 2018).
23. R. V. Lenth, emmeans: Estimated marginal means, aka least-squares means (2022), (available at https://CRAN.R-project.org/package=emmeans).
24. B. Langmead, S. L. Salzberg, Fast gapped-read alignment with Bowtie 2. *Nat. Methods*. **9**, 357–359 (2012).
25. P. Danecek, J. K. Bonfield, J. Liddle, J. Marshall, V. Ohan, M. O. Pollard, A. Whitwham, T. Keane, S. A. McCarthy, R. M. Davies, H. Li, Twelve years of SAMtools and BCFtools. *Gigascience*. **10** (2021), doi:10.1093/gigascience/giab008.
26. S. Tilk, A. Bergland, A. Goodman, P. Schmidt, D. Petrov, S. Greenblum, Accurate allele frequencies from ultra-low coverage pool-seq samples in evolve-and-resequence experiments. *G3 Genes|Genomes|Genetics*. **9**, 4159–4168 (2019).
27. E. K. H. Ho, M. Bartkowska, S. I. Wright, A. F. Agrawal, Population genomics of the facultatively asexual duckweed *Spirodela polyrhiza*. *New Phytol.* **224**, 1361–1371 (2019).
28. D. E. Wood, J. Lu, B. Langmead, Improved metagenomic analysis with Kraken 2. *Genome Biol.* **20**, 257 (2019).
29. J. Lu, F. P. Breitwieser, P. Thielen, S. L. Salzberg, Bracken: estimating species abundance in metagenomics data. *PeerJ Comput. Sci.* **3**, e104 (2017).
30. M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).
31. B. J. Callahan, P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. A. Johnson, S. P. Holmes, DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods*. **13**, 581–583 (2016).
32. C. Quast, E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, F. O. Glöckner, The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* **41**, D590–6 (2013).
33. L. Guillou, D. Bachar, S. Audic, D. Bass, C. Berney, L. Bittner, C. Boutte, G. Burgaud, C. de Vargas, J. Decelle, J. Del Campo, J. R. Dolan, M. Dunthorn, B. Edvardsen, M. Holzmann, W. H. C. F. Kooistra, E. Lara, N. Le Bescot, R. Logares, F. Mahé, R. Massana, M. Montresor, R. Morard, F. Not, J. Pawlowski, I. Probert, A.-L. Sauvadet, R. Siano, T. Stoeck, D. Vaulot, P. Zimmermann, R. Christen, The Protist Ribosomal Reference database (PR2): a catalog of unicellular eukaryote small sub-unit rRNA sequences with curated taxonomy. *Nucleic Acids Res.* **41**, D597–604 (2013).
34. P. J. McMurdie, S. Holmes, phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*. **8**, e61217 (2013).
35. P. Dixon, VEGAN, a package of R functions for community ecology. *J. Veg. Sci.* **14**, 927–930 (2003).
36. M. Schäfer, C. Brütting, I. T. Baldwin, M. Kallenbach, High-throughput quantification of more than 100 primary-and secondary-metabolites, and phytohormones by a single solid-phase extraction based sample preparation with analysis by UHPLC–HESI–MS/MS. *Plant Methods*, **12**, 1-18 (2016).

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Competing interests
Authors declare that they have no competing interests.

Data availability
Raw sequencing data is available on NCBI SRA under Bioprojects PRJNA849359 (pool-seq), PRJNA893536 (16S amplicon metagenomics), and PRJNA893537 (18S amplicon metagenomics). Code and data to replicate analyses are available at:
https://github.com/amalacrino/malacrino_et_al_rapid_plant_adaptation_to_herbivory