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Plant defence to sequential attack is adapted to prevalent herbivores

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Plants have evolved plastic defence strategies to deal with the uncertainty of when, by which species and in which order attack by herbivores will take place[1–3]. However, the responses to current herbivore attack may come with a cost of compromising resistance to other, later arriving herbivores. Due to antagonistic cross-talk between physiological regulation of plant resistance to phloem-feeding and leaf-chewing herbivores[4–8], the feeding guild of the initial herbivore is considered to be the primary factor determining whether resistance to subsequent attack is compromised. We show that, by investigating 90 pairwise insect–herbivore interactions among ten different herbivore species, resistance of the annual plant *Brassica nigra* to a later arriving herbivore species is not explained by feeding guild of the initial attacker. Instead, the prevalence of herbivore species that arrive on induced plants as approximated by three years of season-long insect community assessments in the field explained cross-resistance. Plants maintained resistance to prevalent herbivores in common patterns of herbivore arrival and compromises in resistance especially occurred for rare patterns of herbivore attack. We conclude that plants tailor induced defence strategies to deal with common patterns of sequential herbivore attack and anticipate arrival of the most prevalent herbivores.

During 400 million years, insect herbivores have been driving the evolution of plant defences[9,10]. Individual plants are typically attacked by multiple species of insects that arrive at different moments during a plant's lifetime. The occurrence of insect herbivores may be uncertain in terms of when, by which species, and in which order, the attack will take place. To save costs of maintaining defences in the absence of herbivores, plants alter their level of defence in response to actual herbivore attack[1–3]. These induced defences also allow plants to tailor resistance to the specific attacker which can be recognized by its damage pattern and compounds in the herbivore's oral secretion[3,6]. However, the specific response to one herbivore may compromise the resistance to other herbivores. Resource allocation trade-offs, physiological limitations in the regulation of resistance to sequential herbivore attack, as well as manipulation of induced plant responses by herbivores, may make plants more susceptible to secondary herbivore attack[4–8].

Although plants are known to rapidly evolve to combinations of species they interact with[11,12], we know surprisingly little about the link between induced responses to herbivory and the odds of sequential herbivore attack that plants may have to deal with. The understanding of plant responses to attack by two sequentially arriving herbivore species is built on a large collection of studies that arbitrarily selected pairs of herbivores to study plant resistance to sequential attack[13,14] and in-depth studies of the underlying molecular mechanisms of plant physiological responses to dual attack[3,15–17]. These studies demonstrated that plant responses to attack by leaf-chewing herbivores such as caterpillars are primarily regulated through the jasmonic acid (JA) signalling pathway and generally result in induced resistance to subsequent attack by other leaf chewers[18,19]. Plant responses to phloem-feeding herbivores such as aphids, primarily involve the salicylic acid (SA) signalling pathway and generally result in induced resistance to attack by other phloem-feeding herbivores[20]. However, a sequence of phloem-feeding and leaf-chewing herbivores compromises physiological responses to the later arriving species because of antagonistic cross-talk between the SA and JA pathways[7,8,16,17,21]. The current consensus is that plant responses to initial herbivore attack strongly determine the potential to respond to subsequent herbivory and result in compromises of resistance to sequential attack by herbivores of different feeding guilds[8,14]. This consensus, based on physiological studies, has largely ignored the apparent negative ecological consequences of induced susceptibility to future herbivory[5,7].

Empirical ecological studies and meta-analyses that compared interactions between several pairs of herbivores revealed high specificity in the magnitude of induced resistance or susceptibility in pairwise interactions[13,14]. In addition to feeding guild, diet breadth in terms of the level of food-plant specialization by herbivores is an important trait determining the nature of induced plant responses as well as the susceptibility of herbivores to the induced plant phenotype[13,22]. Whereas the feeding behaviour and growth of generalist herbivores is strongly affected by food-plant specific classes of chemical defences, specialist herbivores can cope with low concentrations of these compounds but are affected by high concentrations[23]. Interestingly, closely related plant species differ in their responses and level of resistance to sequential attack by the same pair of herbivore species[21]. The apparent plant species-specific responses to sequences of attackers indicate differences in selection pressures by sequential herbivory and suggest that plants are not systematically impaired in responding to sequential attack by herbivores of different feeding guilds. We argue that plant responses to initial herbivory should not result in substantial compromises in resistance to subsequent attack by the most prevalent and damaging herbivore species. In optimizing their defence strategy, plants may prepare for likely future attack as part of their induced response to current attack[24].

Our study investigates whether the level of induced resistance to sequential herbivory in the annual plant *Brassica nigra* corresponds with the likelihood of the order and nature of sequential insect attack in the field. We link observations of the order of arrival of

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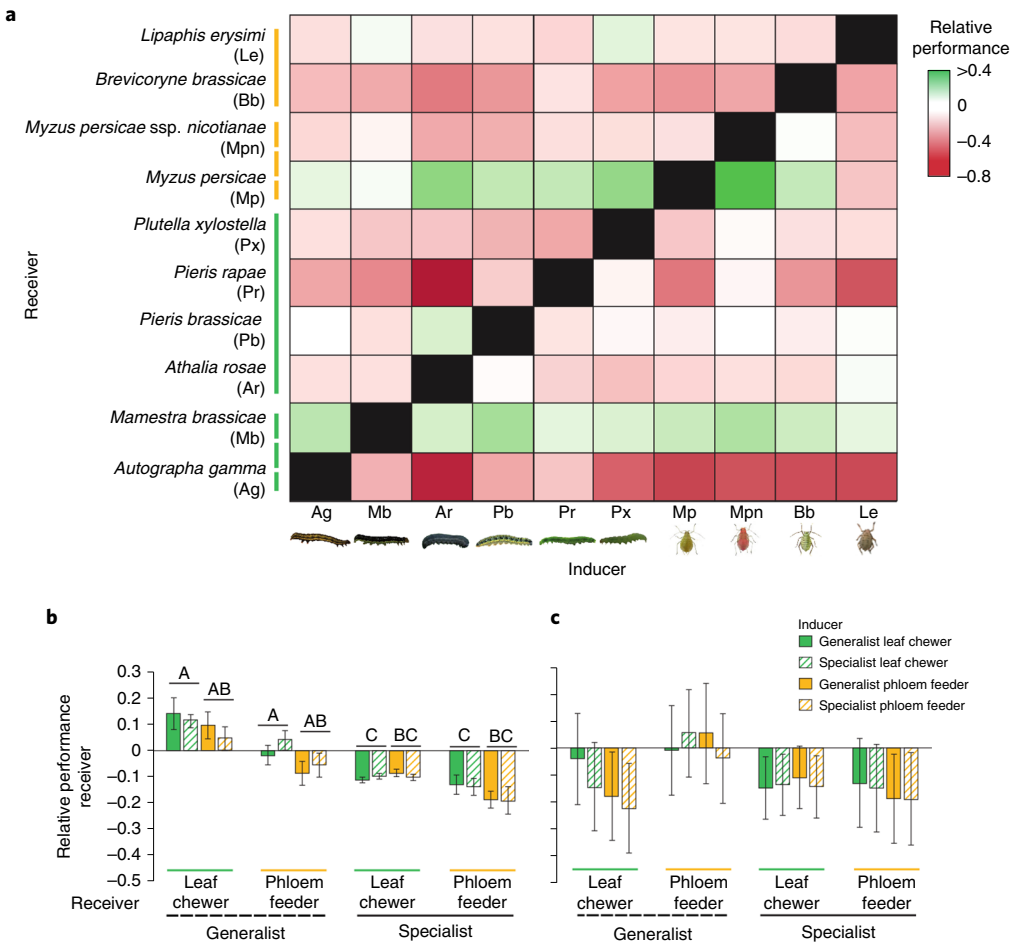

Fig. 1 | Effect of herbivory by ten primary herbivores (inducers) on the performance of the same ten species as receiving herbivores relative to performance on an undamaged control plant. We modelled the performance (number of aphids or larval weight) of each of the receiving herbivores as a function of the inducing herbivores. We calculated the response ratio for each observation by taking the natural logarithm of the ratio between the observed performance of an individual belonging to each species on a treatment and the modelled mean performance of all individuals of that species on control plants. The log response ratio (LnRR) is a dimensionless measure widely used in meta-analysis[26]. **a**, Heat map as visual representation of the effect of each inducer on the relative mean performance of each receiving herbivore. **b, c**, Relative performance of receiver on the basis of the feeding guild and diet breadth of the inducer. Bars represent average relative performance ± s.e.m. ($n = 3{,}315$). Different letters indicate significant differences among treatments in posthoc analyses (LMM). **b**, Estimations from a model that did not include identity of the herbivore species as dependent structure in the model. **c**, Corrected for herbivore species identity by including the herbivore species as random intercepts (Supplementary Tables 9 and 10). See Extended Data Fig. 3 for raw data, including the intraspecific interactions. We left intraspecific interactions out of the analyses, since we specifically aim to identify whether functional traits of the inducing herbivore determine the subsequent ecological outcome of plant–herbivore interactions with a different herbivore. Including plant-mediated interactions among conspecifics would make up 10% of the interactions tested, potentially biasing our results that emphasize specificity caused by feeding guild or diet breadth.

insect herbivores during 3 years of season-long insect community assessments on 488 plants, to a full-factorial assessment of induced resistance in pairwise plant-mediated interactions between ten herbivore species. We specifically test whether induced resistance of the second herbivore can be predicted by traits (feeding guild and diet breadth) of the inducing herbivore or the secondary receiving herbivore species, as well as the commonality of those species and their pairwise interactions in the field (Extended Data Fig. 1 and Supplementary Table 1).

The ten herbivore species induced species-specific plant responses as characterized by the expression of *LIPOXYGENASE 2* (*LOX2*) as marker gene for the JA pathway and *PATHOGENESIS-RELATED PROTEIN 1* (*PR1*) as a marker for the SA pathway (Supplementary Table 2)[3,25]. As predicted from the literature[22,26], chewing herbivores induced a JA response indicated by *LOX2* expression levels and suppressed SA responses indicated by *PR1* expression levels, while phloem feeders elicited only a marginal response in both marker genes (Extended Data Fig. 2). These differences between feeding guilds were most apparent for specialist herbivores that also induced stronger *LOX2* expression than for generalist herbivores (Supplementary Tables 3–6). However, the traits of the inducing herbivore (feeding guild and diet breadth) did not predict the performance of receiving herbivores feeding on the induced plants (Fig. 1 and Supplementary Tables 7 and 8). Performance of receiving herbivores was either almost consistently promoted or consistently inhibited on plants previously induced by herbivores, independent of which herbivore species served as inducer (compare rows in Fig. 1a). Surprisingly, we found much less consistency from the inducing herbivore perspective, as specific inducers could promote or inhibit performance of subsequent herbivores, even when those were of the same feeding guild (compare columns in Fig. 1a). Thus, induction by leaf-chewing herbivores did not predict

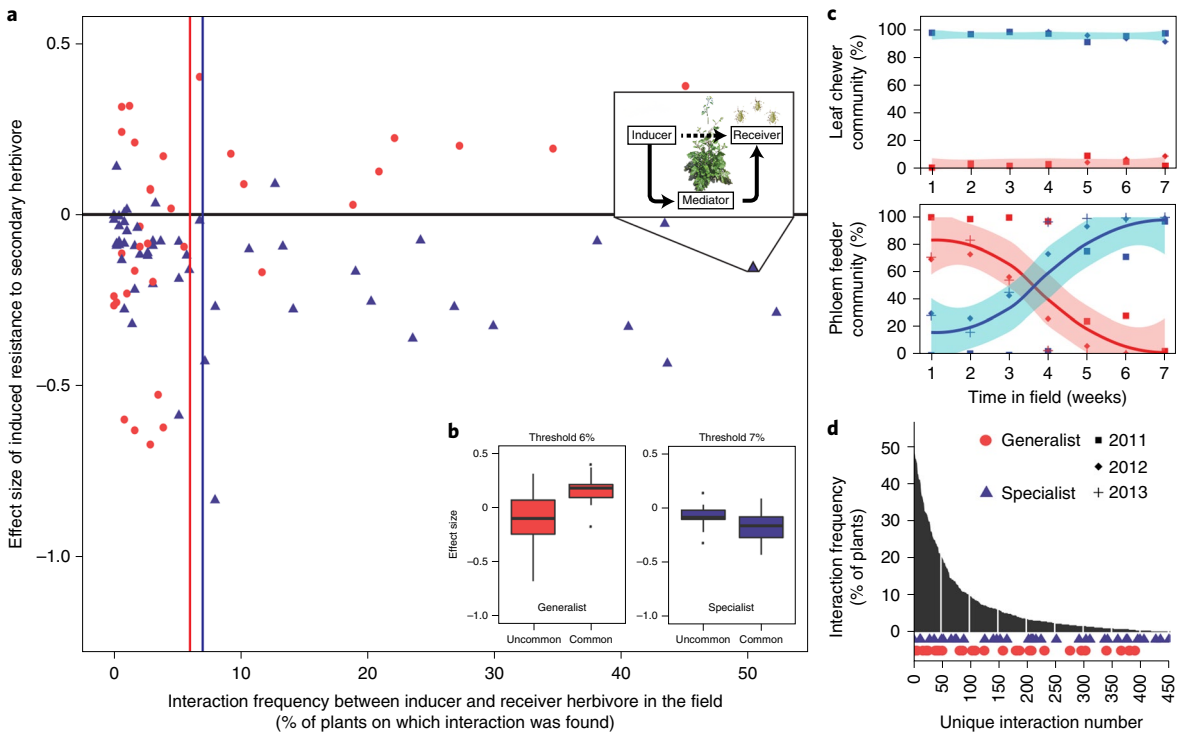

Fig. 2 | Optimal resistance strategies incorporate likelihood of subsequent attack. a, Outcome of pairwise interactions tested in a greenhouse setup related to percentage of plants in our field experiment on which these interactions were observed. The coloured vertical lines represent the threshold set in **b**. **b**, Response ratio of pairwise interactions classified as common or uncommon by an interaction frequency threshold. This threshold was set at 6% or 7% of plants in our field, depending on whether the receiving herbivore was a generalist (left; $n=10$ uncommon, 24 common interactions) or a specialist (right; $n=19$ uncommon, 35 common interactions). These threshold values indicate the percentages of interaction occurrence that yielded clear significant differences between common and uncommon interactions in a sensitivity analysis for 20 threshold values. Response ratio of common and uncommon interactions were significantly different (two-sided Mann–Whitney test; generalist $P=0.004$ and specialist $P=0.002$). The boxes span from the first to the third quartiles, the centre lines represent the median values and the whiskers show the data that lie within the 1.5 interquartile range of the lower and upper quartiles. The datapoints at the ends of the whiskers represent the outliers. **c**, Ratio of specialist and generalist herbivores in the field experiments over time, indicating increasingly specialist-dominated herbivore communities. **d**, The percentage of plants on which the 453 unique interactions observed in the field occurred. Circles and triangles indicate the interactions we tested in our greenhouse setup.

resistance to subsequent feeding by chewers, nor did herbivory by phloem-feeding herbivores predict resistance to subsequent phloem feeders. Moreover, induced responses to phloem feeders did not lead to susceptibility to subsequent feeding by chewing herbivores. The plant phenotype induced by one herbivore species resulted in large variation in both sign and magnitude of effect on performance of the nine other receiving herbivore species (Fig. 1a and Extended Data Fig. 3). These findings are further supported by the absence of a relation between the induced plant phenotype in terms of *LOX2* and *PR1* expression and receiving herbivore performance (Extended Data Fig. 4). Gene expression of *LOX2* and *PR1* upstream in the defence signalling cascade may not predict expression of traits that affect herbivore performance such as primary metabolites, secondary metabolites (glucosinolates) or morphological defences such as trichomes (Supplementary Results and Discussion). Overall, secondary receiving herbivore performance was best explained by diet breadth of the receiving herbivore but was mostly independent of its feeding guild and largely driven by species-specific effects (Fig. 1b,c and Supplementary Tables 7–10). Where most other studies have evaluated up to ten pairwise interactions, we show by using 90 species interactions (excluding intraspecific interactions) that the identity and/or functional group of the inducing herbivore does not affect the performance of a subsequent herbivore on induced plants[14]. These findings ask for a shift of focus in our research field, stepping away from the predictive value currently attributed to the identity or functional group of inducing herbivores when inferring an ecological or evolutionary interpretation of induced plant responses to sequential herbivory. Instead, our data clearly show that the prevalences of the initial and subsequent herbivores in the field are the major determinants of the level of cross-resistance in induced defence strategies.

On the basis of the assembly of insect communities over the lifetime of 488 plants, we found that five out of the 90 pairwise interactions investigated in our greenhouse experiment were not observed in the field. A total of 23 interactions occurred on <1% and 53 interactions were observed on <5% of the 488 plants, while 37 interactions were found on >5% and 11 interactions were observed on >25% of the plants (Fig. 2 and Supplementary Tables 11–14). The frequency of these observed species interactions is determined by overall prevalence of the inducing and receiving herbivore species, as well as by how initial herbivory affects the likelihood of a plant being colonized by a second herbivore species[5]. Our most important finding is that, when assessing the performance of herbivores on previously induced plants relative to their performance on untreated plants in our greenhouse experiment, we observed a reduced performance of specialist herbivores correlated with the increased prevalence of the herbivore under field conditions (Fig. 2a,b). This pattern is inversed when interactions involve a generalist secondary receiving herbivore, showing that common interactions (occurring in >3.5% plants) are associated with higher performance of generalist

secondary receiving herbivores relative to their performance on non-treated plants (Fig. 2a,b). The frequency of interaction pairs to occur was strongly determined by the overall commonality of plant attack by specialist secondary receiving herbivores (Supplementary Table 11). Irrespective of the identity of the initial herbivore attacking the plant, resistance was strongest against prevalent subsequent specialist herbivore species (Extended Data Fig. 5). Even if plants were initially attacked by a prevalent phloem-feeding herbivore species, they were not compromised in resistance to prevalent specialist leaf-chewing herbivores. Thus, plant responses to initial herbivory did not compromise resistance to attack by common subsequent herbivores (Extended Data Fig. 5).

Placing our experimental findings in an ecologically relevant context is imperative for interpretation of the ecology and evolution of induced plant defences. First, the induced response to initial herbivory and enhanced resistance to specialist herbivores is probably adaptive as the insect community on *B. nigra* plants becomes dominated by the most ravaging specialist herbivores as the growing season progresses (Fig. 2c)[27]. Moreover, herbivore-induced *Brassica* plants are more frequently colonized by specialist herbivores than are undamaged plants[5]. Specialist herbivores involved in the most common interactions were also the most prevalent herbivores and colonize individual plants with larger numbers than herbivores in rare interactions (Supplementary Tables 13 and 14). Together, these findings indicate that *B. nigra* plants activate defence strategies to the most likely type of herbivore attack and are not compromised to deal with these attackers by responses to initial herbivory. However, compromises do arise when later arriving herbivores are rare. Second, we observe apparent mismatch of induced plant responses to secondary receiving herbivores with a wide diet breadth (generalists) (Fig. 2a,b). We propose that the costs of this induced susceptibility are relatively small due to the effectiveness of constitutive resistance[28]. Even though relative performance of surviving generalist herbivores on induced plants compared to undamaged plants was positive, the mortality rates were high compared to specialist herbivores and the sizes of those that survived were small (Extended Data Fig. 3). Third, strong negative effects on rare subsequent herbivores can be explained by non-mutually exclusive proposals (Fig. 2a). They may indicate an evolutionary signature of past arms races between *B. nigra* and the specific herbivore species[27]. Alternatively, the strong, induced cross-resistance may indicate resistance to a subsequent herbivore that has strong outbreak cycles that did not occur during the 3-yr period of our field observations[29]. Finally, some of the resistance to uncommon subsequent herbivores may be due to trait overlap with herbivore species involved in common interactions and hence similarity in performance on induced plants.

The results of this study demonstrate that *B. nigra* plants optimize their defence responses to herbivory by anticipating the arrival of the most abundant herbivores in response to initial herbivory. To understand the evolution of induced defence strategies, plant physiological responses to single herbivore attack and its plasticity to multi-herbivore attack should thus be evaluated in a community perspective, including the dynamic patterns in order and timing of multi-herbivore attack. The adaptive value of plant responses to initial attack is largely determined by the predictability of subsequent herbivore attack and herbivore traits such as diet breadth. The cross-talk between SA and JA should not be extrapolated to ecological outcomes of herbivore interactions because substantial variation in plant defence phenotypes may arise after initial expression of genes basal to the JA and SA signal transduction pathways[12]. SA and JA cross-talk in plants may dampen the overly strong induction of specific defensive phenotypes and allow a fine-tuning where the urgent need to respond to current stress is traded off to retain the capability to respond to probable subsequent stresses[8]. We speculate that initial attack by any herbivore species may result in plants mobilizing resistance to tailor future prevalent herbivores. Thus, the simultaneous upregulation of JA- and SA-related genes to initial attack found in many studies on induced responses to individual herbivores could presumably be responses targeted to predictable future attack[24]. For instance, *PR1* expression in response to chewers may predict that plants are exposed to a large likelihood of future aphid attack. Hence, plants optimize their defence strategy to match common patterns of sequential herbivore attack, so as to not compromise responses to the most prevalent herbivore attacks.

Methods

Study system. *B. nigra* (L.) Koch is an annual herbaceous plant common throughout Europe and used as host plant by a wide range of phytophagous insects[30,31]. Seeds collected from a local population in Wageningen, the Netherlands, were germinated in trays. One-week-old plants were transplanted and grown in pots (diameter = 15 cm, volume = 1 l) under greenhouse conditions (22 ± 2 °C, 60–70% relative humidity (r.h.) and light 16 h/dark 8 h). Four-week-old plants were used in greenhouse experiments; seedlings were planted in field experiments.

Ten insect species were used as herbivores (Supplementary Table 1). We used first instar larvae of the leaf chewers: the silver Y, *Autographa gamma* (Ag) (Lepidoptera: Noctuidae); the cabbage moth, *Mamestra brassicae* (Mb) (Lepidoptera: Noctuidae); the large cabbage white, *Pieris brassicae* (Pb) (Lepidoptera: Pieridae); the small cabbage white, *P. rapae* (Pr) (Lepidoptera: Pieridae); the turnip sawfly, *Athalia rosae* (Ar) (Hymenoptera: Tenthredinidae); and second instar larvae of the diamondback moth, *Plutella xylostella* (Px) (Lepidoptera: Plutellidae). Starting mass of these newly hatched herbivores is below the error margins of an analytical balance and was not assessed. We used adult wingless individuals of the phloem feeders: the cabbage aphid, *Brevicoryne brassicae* (Bb); the green peach aphid, *Myzus persicae* (Mp); the tobacco aphid, *Myzus persicae* ssp. *nicotianae* (Mpn); and the mustard aphid, *Lipaphis erysimi* (Le) (all Hemiptera: Aphididae). The insects *A. gamma*, *M. brassicae*, *P. brassicae*, *P. rapae*, *B. brassicae* and *P. xylostella* were reared on Brussels sprouts (*B. oleracea* L. var. *gemmifera* cv. Cyrus), while *A. rosae*, *M. persicae*, *M. persicae* ssp. *nicotianae* and *L. erysimi*, were reared on radish (*Raphanus sativus*) (all from the stock rearing of the Laboratory of Entomology, Wageningen University). The insect cultures were kept under greenhouse conditions (22 ± 2 °C, 50–70% r.h. and light 16 h/dark 8 h). We classified herbivore species as host specialists when their documented trophic niche in the relevant herbivorous life stages is limited to plants of Brassicaceae (Supplementary Table 1). The herbivore species were selected for being culturable, the possibility to measure a proxy for their performance (weight gain or population growth) and to represent a balanced spectrum of feeding guilds, level of food-plant specialization and prevalence in the field.

Experimental design. To assess plant defence responses to herbivory and the ecological outcome of all 90 pairwise interactions among the ten different insect-herbivore species in our experiment, we divided the experiment into ten blocks. In each experimental block, we used all herbivore species as inducers and only one of the ten species as receiving herbivore (receiver) (Extended Data Fig. 1). Each block consisted of 110 plants, with ten plants assigned to each of the herbivore inducer treatments and ten plants were left untreated. Treatments were randomly arranged over two benches in a greenhouse compartment (22 ± 2 °C, 60–70% r.h. and light 16 h/dark 8 h). To prevent desiccation of the plants and herbivores from moving between plants, we placed plants in inundated trays. We infested the youngest fully developed leaf of each plant with either ten wingless aphids or five first instar larvae. All herbivores were left free to move on the plant, allowing them to choose their preferred feeding sites. Undamaged control plants were treated similarly to herbivore-induced plants.

Plant transcriptional responses to herbivore induction. As a measure for plant defensive responses to each of the ten initial herbivores, we quantified levels of transcription of two marker genes in *B. nigra*; *LIPOXYGENASE 2* (*LOX2*) for the JA signalling pathway[32] and *PATHOGENESIS-RELATED PROTEIN 1* (*PR1*) for the SA signalling pathway[33] (Supplementary Table 2). In each experimental block, leaf samples were taken at 24 or 96 h after plants were infested with herbivores. The timepoints for *LOX2* and *PR1* expression were selected on the basis of their peaks of expression after herbivory in *B. nigra* identified in previous studies[34,35]. For each herbivore treatment and timepoint, we sampled the youngest fully developed leaf on which herbivores had been released from five of the plants. These leaves were always damaged and had the most released herbivores still present. Samples were taken by detaching the leaf with a razorblade and punching a leaf disc of 2 cm in diameter with a leaf puncher. Herbivores present on the detached leaf were placed back on the plant. The five leaf discs were combined to constitute one biological replicate, submerged in liquid nitrogen right after sampling and stored at −80 °C until further analysis. Per experimental block, a single biological replicate per inducer and timepoint was obtained, yielding a total of ten biological replicates per treatment and timepoint for the entire experiment. The frozen

samples were ground to a fine powder with a pestle and RNA was isolated from the plant material by using RNeasy Plant Mini Kit (Qiagen) and treated with DNAse I (Invitrogen) following the manufacturer's instructions. After isolation, the RNA concentration was quantified using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies; all samples with optical density of 260 nm / 280 nm of 1.9–2.2 ratio). Samples were diluted and adjusted to an RNA concentration of 50 ng μl^{-1}. From the RNA samples we synthesized complementary DNA, using the SensiFAST cDNA synthesis kit (Bioline). We quantified expression levels of each sample by quantitative PCR with reverse transcription, using the SensiFAST SYBR no-ROX kit (Bioline). A total 5 ng of the cDNA template were added to the reaction with a total volume of 20 µl. The reactions were performed with shuttle PCR conditions using a CFX96 Touch Real-Time PCR Detection System (Biorad). All reactions were conducted using two technical replicates and samples were omitted from further analysis if the difference in expression between the technical replicates was >0.5. Plate setups also included negative controls (no template) and interrun calibrators. Gene-expression data were imported to qBase+ v.3.1 (Biogazelle) to calculate Calibrated Normalized Relative Quantity (CNRQ). The CNRQ value represents the relative quantity (gene-expression level) of a sample for a given target gene corrected by the expression value of two reference genes for each sample. We tested the following reference genes for expression stability: *ACTIN-2* (*ACT2*), *BETA-TUBULINE* (*B-TUB*), *ELONGATION FACTOR-1* (*EF1*), *PEROXIDASE 4* (*PER4*), *SECRETION ASSOCIATED RAS RELATED GTPASE 1A* (*SAR1A*) and *GLYCERALDEHYDE-3-PHOSPHATE* DEHYDROGENASE (*GAPDH*). The last two genes were selected as reference genes because they had highest stability (their expression was not affected by the treatment). Data were corrected for differences between runs using interrun calibrators.

Herbivore performance. To quantify pairwise plant-mediated interactions among the full matrix of interactions between the ten herbivores (90 interactions, excluding intraspecific interactions), we assessed herbivore performance on herbivore-induced plants. These were the same plants that were sampled for gene-expression analyses, allowing for direct correlations between defence gene expression and herbivore performance. Seven days after initial herbivore (inducers) infestation, we removed all herbivores (to exclude direct effects of inducing herbivores on the receiver herbivore and thus isolate plant-mediated effects through herbivore-induced plant responses) and immediately re-infested plants with the second herbivore species (receivers). When removing herbivores, we retrieved inducing herbivores from all plants that were clearly damaged by these herbivores. We placed either 20 wingless aphids or ten first instar larvae of the receiving herbivore species on all plants in a block, repeating the setup with a different herbivore receiver in each block. After 7 d we assessed the performance of the receiving herbivore by either counting the number of aphids or by collecting and weighing larvae individually (scales: Sartorius CP2P, Mettler Toledo ML54/01) (Extended Data Fig. 1). Aphids were counted twice by different people and the average was taken as a measure of their performance. Herbivore performance is commonly used as a parameter of plant resistance to herbivory and provides insight into how initial herbivory results in induced resistance or in susceptibility to receiving herbivores.

Herbivore interaction frequency under field conditions. Information on herbivore interaction frequency under field conditions was collected during 3 yr of common garden experiments (summers of 2011, 2012 and 2013) in an experimental field in Wageningen. We recorded herbivore identity and time of arrival on a total of 540 *B. nigra* plants (180 plants per year). Insect presence was assessed weekly, surveying herbivore community development on individual plants from seedling until seed maturation. Plants that were monitored less than four times were removed from the dataset, retaining 488 plants and 895,236 herbivore observations in the analysis (Supplementary Table 14). We calculated the relative number of plants on which we recorded one herbivore species arriving before or at the same time as another herbivore species. The 90 pairwise interactions tested in our greenhouse experiments spanned the range from common to rare among all pairwise interactions identified on *B. nigra* plants in the field (Fig. 2d).

Statistical analysis. Data obtained in the greenhouse experiment and the 3 yr of field observations were aggregated and analysed as a single independent experiment. We did not use confirmatory analysis techniques to test our models to a separate dataset. For each modelling analysis, we selected the best model from a set of candidate models by comparison of the Akaike information criteria (AIC) with a selection threshold of $\Delta 5$ AIC[36]. Candidate models differed in the number of fixed factors (and their interactions) and the stochastic distribution. Full models included all interactions between fixed factors and in case mixed models were applied they included all random intercepts. When mixed effect models were applied, we first optimized the random structure and then the fixed structure of the model[37]. When more than one model was optimal on the basis of our AIC threshold, we explored all optimal models to verify that our interpretation of the results would not differ among models and reported the model with lowest AIC. We further compared the AIC of optimal models with the AIC of null models which did not contain any predictors (that is, the model assumes the effect of all fixed factors is equal to zero). Parameters of the optimal model were estimated by restricted maximum likelihood estimation or Laplace approximation. After model selection, we evaluated the significance of different factors in the optimal (generalized) linear (mixed) model (next two sections). Pairwise posthoc comparisons were evaluated by Tukey's honest significant difference (HSD) test and contrasts were considered significantly different at $P \leq 0.05$. Statistical analyses were done using the nlme[38], lme4[39], lmtest[40] and emmeans[41] packages in R (v.3.2.4, R Core Team[42]).

Plant transcriptional responses to herbivore induction. To model species-specific effects of herbivore induction on the relative expression of *LOX2* and *PR1* we applied generalized linear models (GLMs) with gamma error distribution and log link function. The full models included herbivore species identity and the timepoint at which the sample was taken (24 or 96 h after induction) as explanatory factors. To detect broader patterns on the basis of the feeding behaviour of the herbivores, we performed a second analysis. Here, we applied generalized linear mixed models (GLMMs) with gamma error distribution and inverse link function to evaluate effects of the inducing herbivore's feeding guild (phloem feeder or chewing herbivore), its level of host specialization (specialist or generalist) and the timepoint at which the sample was taken (24 or 96 h after induction) on the relative expression of *LOX2* and *PR1*. To interpret patterns in transcription levels in terms of feeding guild and host specialization of herbivores while correcting for variable species effects within feeding guild and host specialization, species identity was taken as a random effect. As we were interested in species-specific effects on relative gene expression and exploring patterns on higher functional levels (herbivore feeding guild and host specialization) rather than changes in expression over time, we performed pairwise comparisons for the two timepoints separately.

Herbivore performance. To allow comparison of performance across all herbivore species, we calculated the response ratio (LnRR) for each observation (number of aphids or larval weight) by taking the natural logarithm of the ratio between the observed performance (X) of an individual (i) belonging to species (S) on treated plants (T) and the mean performance of species (S) on control plants (C):

LnRR = ln(individual performance on induced plant/average species performance on control plants)

$$\mathrm{LnRR} = \ln[X_{iST}/(X_{SC})]$$

The response ratio is a practical measure when comparing strength and sign of treatment effects across multiple experiments and different response variables and is widely used in ecological meta-analysis[26,43]. To get robust estimates of mean performance on control plants, we fitted mixed effect models expressing the herbivore performance (number of aphids or larval weight) as a function of the induction treatment and including the plant individual and the timepoint at which the leaf sample was taken as random factors. The estimated means on control plants were used as denominators in the response ratios. We applied linear mixed effect models (LMMs) for each of the phloem-feeding herbivores and *P. brassicae* and GLMMs with gamma error distribution and log link function for each of the remaining leaf-chewing herbivores. We found heterogeneity of variance in the models estimating phloem feeder performance and in the model estimating *P. brassicae* performance. To adjust for this, we allowed variance to be different for each of the inducing herbivores.

Next, we evaluated if herbivore performance depended on the inducing or receiving herbivore. We applied an LMM with response ratio as dependent variable and inducing and receiving herbivore identity as independent variables. We corrected for dependency between observations by including the plant individual as random intercept and adjusted for heterogeneity of variance by allowing variance to be different for each of the receiving species. The alternative model that was tested was a model that related the response ratio to the feeding guild (phloem feeder or chewing herbivore) and level of host specialization (specialist or generalist) of the inducer and receiver herbivores with plant individual as random effect. We excluded pairwise interactions between the herbivores themselves, and between *M. persicae* ssp. *nicotianae* and *M. persicae* from this analysis, as for the latter the data obtained were outliers in effect size. Although including this interaction did not significantly change the interpretation of our results, it was influential in determining confidence intervals and AIC values, hampering convergence of analysis of the full matrix of interactions. In a third analysis, we added a random effect including species identity of both the inducing and receiving herbivore to the model evaluating feeding guild and level of host plant specialization.

In a final analysis, we assessed the role of prevalence of both the inducing and receiving herbivores as observed under field conditions in predicting the performance of the receiving herbivore. In these models, the estimated response ratios of pairwise interactions were included as the dependent variable and the feeding guild (phloem feeder or chewing herbivore), level of host specialization (specialist or generalist) and prevalence of the inducer and receiver herbivores were included as explanatory variables. The backward selection procedure applied to optimize the model converged on an optimum that included a complex four-way interaction. Hence, to avoid overinterpretation of our data, we chose to optimize the model by applying a forward stepwise selection procedure.

Relation to field observations. To evaluate whether patterns in herbivore performance under greenhouse conditions could be linked to interaction

frequency under field conditions, we related the frequency of each tested pairwise interaction observed in the field to their estimated mean response ratio in the performance assays. We annotated interactions on the basis of host specialization of the receiving herbivore (host specialists or generalists) and analysed the two groups separately. We classified interactions as either common or uncommon for a range of 20 threshold values, on the basis of the percentage of plants on which interactions were observed (interaction frequency). To statistically compare the mean response ratio of common and uncommon interactions at each of the 20 threshold values, we applied a non-parametric rank-based test (Mann–Whitney). To assess variability of the observed effect size, independent of the sign of the effect, we transformed the estimated mean response ratio by taking its absolute value. This transformed dataset was subjected to the same non-parametric analysis with 20 threshold values of interaction frequencies used in our previous analysis. As the number of common or uncommon interactions was dependent on the assessed interaction frequency threshold, we compared the variance of the group with the least number of pairwise interactions with the variance of an equal number of randomly selected interactions from the largest group. This permutation procedure was repeated 1,000 times for each assessed threshold level. We used prevalence of herbivores (the number of plants colonized by a specific species) as a measure for the importance of interactions, instead of the abundance of the herbivore in the field (number of individuals counted over all plants). Abundance of herbivores on an individual plant may not accurately compare fitness costs of herbivory across species, since the costs of attack by many aphids may not compare to attack by a single ravaging caterpillar.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data are available in the main text, the Supplementary Information and on the DRYAD public repository (https://doi.org/10.5061/dryad.pnvx0k6n3).

Code availability

All R code used in our analysis is made available on the DRYAD public repository (https://doi.org/10.5061/dryad.pnvx0k6n3).

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Author contributions

E.H.P., D.M. and M.F.B. planned and designed the study and developed the methodology. E.H.P., D.M., M.F.B., Q.R. and J.B. performed the experiments. M.F.B. and J.B. analysed gene expression. D.M. and B.D. analysed performance and field data. All authors contributed to writing of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41477-021-00999-7.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41477-021-00999-7.

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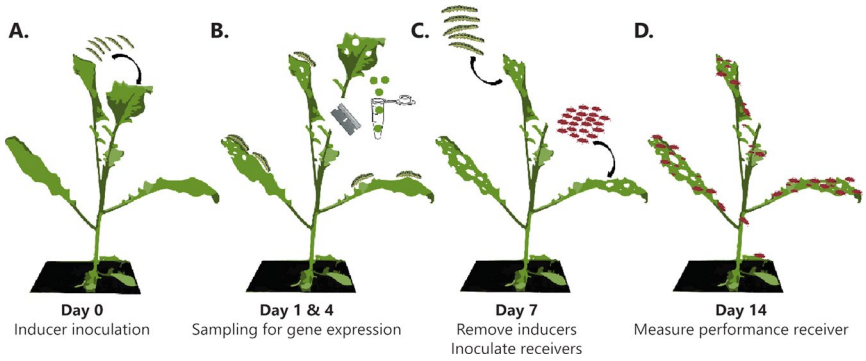
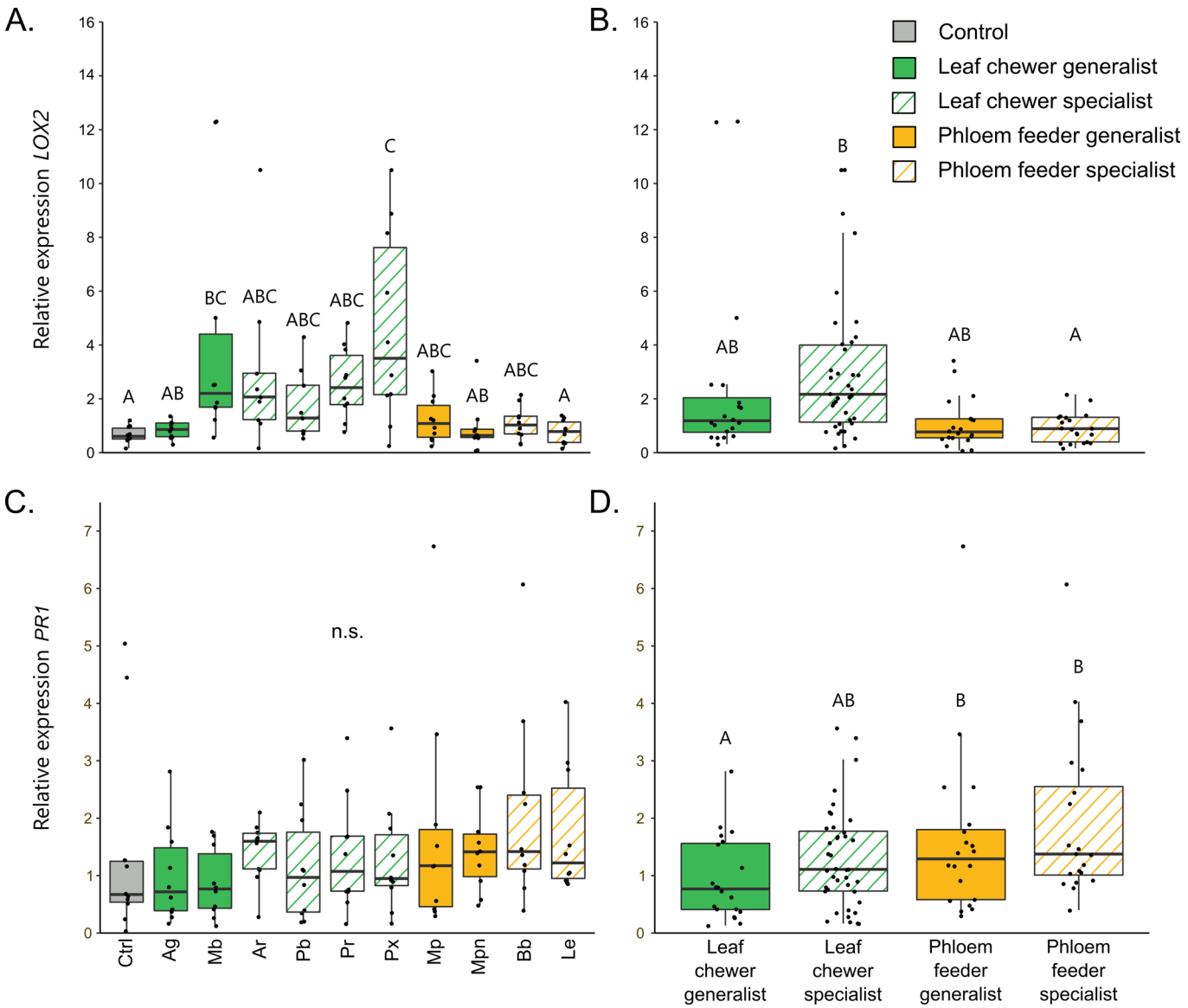
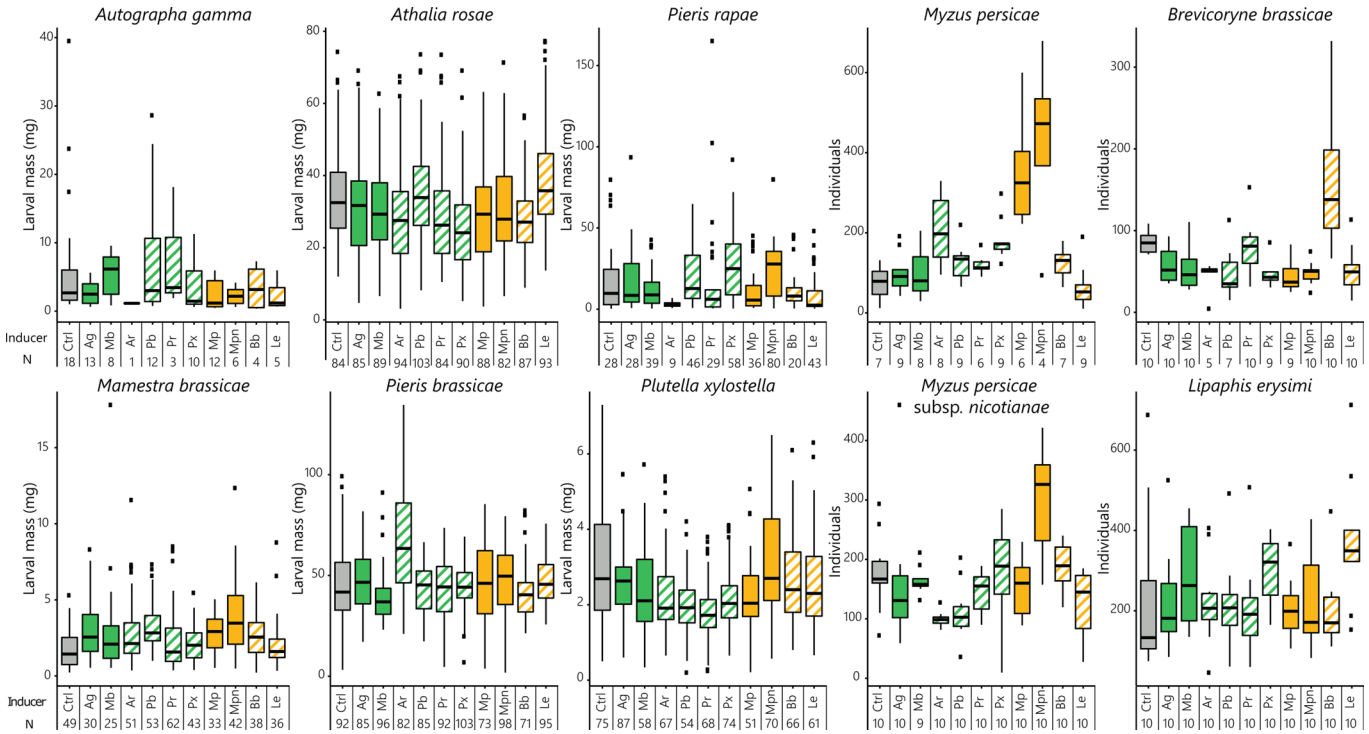


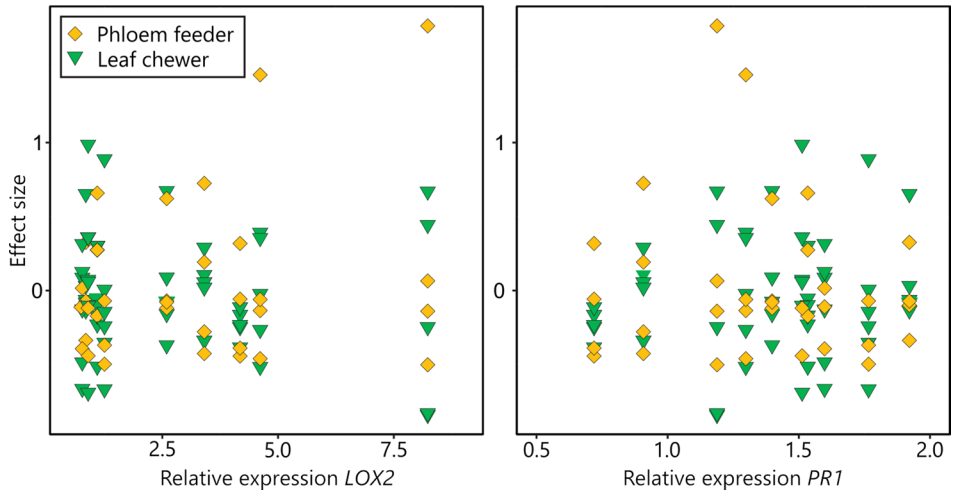
Extended Data Fig. 1 | Experimental setup for the performance experiment. A, *Brassica nigra* plants were infested with one of ten insect species as primary herbivores (inducers). Five leaf chewers or ten phloem feeders were used as inducers. **B**, One and four days after plant infestation with herbivores, leaf samples for gene-expression analyses were taken (each plant was sampled only once). **C**, Seven days after plant infestation with herbivores, all remaining inducers were removed and plants were infested with receiving herbivores. Ten leaf chewers or 20 phloem feeders were used as receivers. **D**, Seven days after plant infestation with receivers, their performance was measured (leaf chewer weight or number of phloem feeders). We repeated this setup ten times, each time using a different insect as receiver, and preparing ten plant replicates per treatment (Supplementary Table S1).



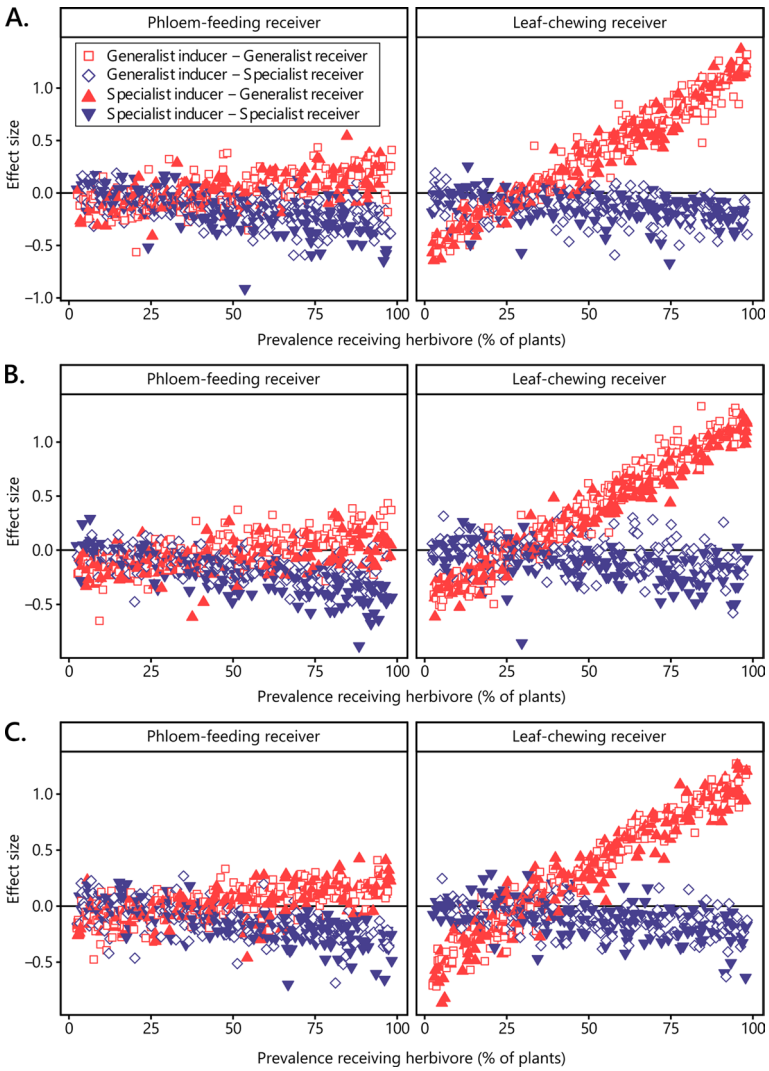
Extended Data Fig. 2 | Expression levels of the JA biosynthesis gene *LOX2* and the SA responsive gene *PR1* on *Brassica nigra* leaves after 96 h of herbivory. Herbivores used were the leaf chewers *Autographa gamma* (Ag), *Mamestra brassicae* (Mb), *Athalia rosae* (Ar), *Pieris brassicae* (Pb), *P. rapae* (Pr) and *Plutella xylostella* (Px), and the phloem feeders *Myzus persicae* (Mp), *M. persicae* ssp. *nicotianae* (Mpn) *Brevicoryne brassicae* (Bb) and *Lipaphis erysimi* (Le). **A**, Effects of herbivory on the relative expression of *LOX2* for each herbivore species. **B**, Effects of herbivory on the relative expression of *LOX2* for herbivores grouped by feeding guild and diet breadth. **C**, Effects of herbivory on the relative expression of *PR1* for each herbivore species. **D**, Effects of herbivory on the relative expression of *PR1* for each herbivore species grouped by feeding guild and diet breadth. The boxes span from the first to the third quartiles, the centre lines represent the median values, and the whiskers show the data that lie within the 1.5 interquartile range of the lower and upper quartiles. The datapoints at the ends of the whiskers represent the outliers. Different letters refer to significant differences at $P < 0.05$ based on GLM and Tukey HSD tests adjusting for multiple comparisons (Supplementary Table 3–6), whereas n.s. indicates no significant difference in post-hoc comparisons was found. Each treatment in panels A and C is supported by n = 10 each, while bars in panels B and D are based on n = 40 for specialist chewing herbivores, or n = 20 for the other feeding guild by host specialization combinations.



Extended Data Fig. 3 | Performance of secondary receiving herbivores on *Brassica nigra* plants induced by one of ten different herbivore species. Larval mass or number of individuals on plants induced by herbivory from the leaf chewers *Autographa gamma* (Ag), *Mamestra brassicae* (Mb), *Athalia rosae* (Ar), *Pieris brassicae* (Pb), *P. rapae* (Pr) and *Plutella xylostella* (Px), and from the phloem feeders *Myzus persicae* (Mp), *M. persicae* ssp. *nicotianae* (Mpn) *Brevicoryne brassicae* (Bb) and *Lipaphis erysimi* (Le), and on non-treated plants (Ctrl). n = 4053. Panels correspond to the different receiving herbivores and the numbers below indicate the recapture rate for chewing herbivores, or the number of plants on which phloem feeder populations were assessed. The boxes span from the first to the third quartiles, the centre lines represent the median values, and the whiskers show the data that lie within the 1.5 interquartile range of the lower and upper quartiles. The datapoints at the ends of the whiskers identify the outliers.



Extended Data Fig. 4 | Performance of herbivores is not explained by marker gene expression. Performance of receiving herbivores (effect size; the natural logarithm of the ratio between individual performance on induced plant / average species performance on control plants) related to the relative expression of the JA-marker gene *LOX2* (left panel) or the SA-marker gene *PR1* (right panel). Colours and shapes correspond to the feeding guild of the receiving herbivore.



Extended Data Fig. 5 | Resistance strategies are predicted by prevalence of the receiving herbivore. Model predictions of performance of receiving herbivores (effect size; the natural logarithm of the ratio between individual performance on induced plant / average species performance on control plants) related to the prevalence of the receiving herbivore, separated for phloem-feeding herbivores (left panels) and leaf-chewing herbivores (right panels). Colours and shapes correspond to combinations of diet breadth of the inducing and receiving herbivores. **A**, Model predictions did not constrain the prevalence of the inducing herbivore. **B**) predictions were constrained in prevalence of the inducing herbivore at 2.5% of plants. **C**) predictions were constrained in prevalence of the inducing herbivore at 50% of plants.

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	no software was used
Data analysis	Statistical nalyses were done using the nlme (version 3.1-131.1), lme4 (version 1.1-17), lmtest (version 0.9-36), and emmeans (version 1.2.1) packages in R (v3.2.4, R Core Team 2016). All packages are published and refered to in our reference list.

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All studies must disclose on these points even when the disclosure is negative.

Study description	The study consisted of greenhouse and field experiments. Greenhouse experiments: We measured larval performance by assessing mass after 7 days of feeding. For all insect herbivores we could use newly hatched larvae of the same batch form our stock cultures. These larvae have similar starting mass that is below the error of an analytical balance and thus starting mass was not assessed. Aphid population growth was measured by introducing 20 aphids and measure population size after 7 days by counting the number of apids (adults and nymphs). 10 larvae were placed on each of the 10 individual plant replicates per treatment. Thereby we account for both plant and herbivore variation (used as random effect in the models) Each of the 10 herbivore species was measured for its performance on plants previously damaged by all other herbivores and an undamged plant that served as control. Each herbivore assessment was done as a separate block and effect sizes across blocks were compared through meta-analysis. Field experiment: We monitored natural occurance of insects on a total of 540 Brassica nigra plants, 180 for each of the three study years (2011-2013). Insect presence was monitored on a weekly basis to infer order of arrival of herbivore species over the lifetime of plants in the field.
Research sample	Greenhouse experiments: We selected 10 insect herbivore species to include replication of both chewing (6) and phloem feeding (4) herbivores, as well as their level of food plant specialisation (generalist, specialist). These herbivores were selected based on being part of the natural community on Brassica nigra plants as well as the possibility to culture the herbivores in large enough numbers for experiments. Performance of each of these herbivores was assessed on 10 individual plants of a treatment to account for plant and herbivore variation. Field experiments: We could monitor 180 plants per season based on a 40 hour work week of a single researcher for a total of 4.5 months per year. This was done to maximise resolution of field observations on natural colonisation of plants by insects across the season.
Sampling strategy	Sample size was not predetermined by calculations, but by maximal size for feasibility and within these boundaries the maximal replication to cover plant and herbivore variation. Based on our extensive experience with herbivore responses to induced plants, we know that effects can be reveled by sampling mass of 50-100 individual herbivores or 10 plants for aphid population growth. We follow the same sampling effort as in our previous work: Fernández de Bobadilla, M., Bourne, M. E., Bloem, J., Kalisvaart, S. N., Gort, G., Dicke, M., & Poelman, E. H. (2021) Insect species richness affects plant responses to multi-herbivore attack. New Phytologist. doi:10.1111/nph.17228
Data collection	Greenhouse experiments: Due to the size of the experiments, five researchers (EHP, DM, MFB, QR and JB) performed the experiments. Per experimental block, the five researchers prepared the experiments for herbivore infestation, leaf sample collection for gene expression and counting aphid populations size and collecting herbivores for assessment of their mass. A single researcher weighed all herbivores individually on the same balance within a single day. Chewing herbivores were weighed using a microbalance (Mettler Toledo ML54/01) and both caterpillar weight and aphid counts were recorded using pencil and paper. Field experiments: Each of the three years the monitoring of the insect community over the season was done by 1 observer with ample knowledge on insect identification in the study system. Each plant was monitored for insect presence by inspecting all plant organs for insect presence. Due to repeated observations on the same plant over the season, no insects were collected. Field observations were recorded using pencil and paper.
Timing and spatial scale	Greenhuse experiments: The greenhouse experiments were conducted over a period of 2 years with short intervals between blocks when greenhouses were cleaned, or during peak temperatures in the summer when climate control may not function optmially. Each experimental block from planting until herbivore measurement lasted for 6 weeks. All blocks were conducted between January 2017 and December 2018. Field experiments: Each of the three years (2011-2013) experiments were conducted following the same protocol and study design. Plants were planted in calender week 21 (end of May) and monitored for insect presence until plants scenessed end of September.
Data exclusions	Field experiments: To accurately assess order of arrival of insect herbivores, we omitted individual plants that were monitored less than 4 times, retaining 488 plants and 895,236 herbivore observations in the analysis.
Reproducibility	Greenhouse experiments: Out of the total of 90 interaction pairs tested in the greenhouse, we repeated the same interaction pairs in separate experiments and research projects. All outcomes of these experiments were similar to the findings in our full set of 90 interaction pairs. Due to the large effort to test all these 90 interaction pairs (2 years of work involving 5 persons full time in each experimental block) we could only replicate 5% of the experiments. The outcomes confirmed earlier findings. To confirm accuracy of aphid counts, two persons were counting the same plants for aphid population size and numbers were averaged for the two counts. Field experiments: The field experiments were replicated across 3 years to account for annual variation.
Randomization	Greenhouse experiments: Performance of each herbivore species was measured in separate blocks. Within blocks the treatments

Randomization	were randomly distributed in the greenhouse. Random effects were used for larvae that were sampled from the same plant. Field experiments: seedlings were randomly assigned to their position on the field site.
Blinding	Blinding of assessment of herbivore performance was achieved by using unique numbers for samples that were brought to the person weiging the larvae or the persons assessing the number of aphids. Investigators collected data from plants at predetermined locations in the greenhouse compartment. As treatments were randomised, investigators were unbiased in terms of the treatments they collected data from. However, treatment could not be fully blinded for investigators collecting the data, since they could recognise the insect species on plants.

Did the study involve field work? ☒ Yes ☐ No

Field work, collection and transport

Field conditions	We performed common garden experiments on the same study site for 3 years, replanting each year. The site is an organic farm part of the experimental field station of Wageningen University. The experiments were conducted from May-October in 2011-2013, spring and summer). The study site was located in Wageningen, The Netherlands, on a predominantly sandy soil, with climate conditions matching a temporal maritime climate. Netherlands average temperatures in June - August 2011: 16.3°C, 2012: 16.9°C, 2013: 17.5°C; average precipitation June - August 2011: 350 mm, 2012: 286 mm, 2013: 137 mm. Data made available by the Royal Netherlands Meteorological Institute.
Location	The field site is located in Wageningen, the Netherlands (latitude: 51.99064156; longitude: 5.6619072; elevation 11m)
Access & import/export	No permits were required to work with the native plant species (Brassica nigra) and natural presence of insects visiting these plants.
Disturbance	No disturbance to natural ecosystems were caused. The field is part of an agricultural and managed ground.

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Methods

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Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	We used lower invertebrate organisms (insects: all female aphids and caterpillars of mixed sex) originating from stock cultures collected in the experimental fields of Wageningen University (wild populations; unspecified strains). Ten insect species were used as herbivores. We used first instar larvae of the leaf chewers: Autographa gamma (Lepidoptera: Noctuidae); Mamestra brassicae (Lepidoptera: Noctuidae); Pieris brassicae (Lepidoptera: Pieridae); P. rapae (Lepidoptera: Pieridae); Athalia rosae (Hymenoptera: Tenthredinidae); and second instar larvae of the diamondback moth, Plutella xylostella (Lepidoptera: Plutellidae). We used adult wingless individuals of the phloem feeders: Brevicoryne brassicae; Myzus persicae; Myzus persicae sub. nicotianae; Lipaphis erysimi (all Hemiptera: Aphididae). The insects A. gamma, M. brassicae, P. brassicae, P. rapae, B. brassicae and P. xylostella were reared on Brussels sprouts (Brassica oleracea L. var. gemmifera cv. Cyrus), while A. rosae, M. persicae, M. persicae sub. nicotianae and L. erysimi, were reared on radish (Raphanus sativus). The insect cultures were kept under greenhouse conditions (22 ± 2 °C, 50–70 % r.h. and L16:D8).
Wild animals	We monitored occurance of lower invertebrates by visual observation in the field
Field-collected samples	the study did not involve samples collected in the field
Ethics oversight	No ethical approval or guidance was required. Research on insects (lower invertebrates) is not under ethical approval.

Note that full information on the approval of the study protocol must also be provided in the manuscript.