

EVOLUTIONARY BIOLOGY

Rapid plant evolution driven by the interaction of pollination and herbivory

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Pollination and herbivory are both key drivers of plant diversity but are traditionally studied in isolation from each other. We investigated real-time evolutionary changes in plant traits over six generations by using fast-cycling *Brassica rapa* plants and manipulating the presence and absence of bumble bee pollinators and leaf herbivores. We found that plants under selection by bee pollinators evolved increased floral attractiveness, but this process was compromised by the presence of herbivores. Plants under selection from both bee pollinators and herbivores evolved higher degrees of self-compatibility and autonomous selfing, as well as reduced spatial separation of sexual organs (herkogamy). Overall, the evolution of most traits was affected by the interaction of bee pollination and herbivory, emphasizing the importance of the cross-talk between both types of interactions for plant evolution.

Insects and plants have been interacting since their origins, with these interactions driving the evolution of their notable diversity (1). Insects impose strong selection on plants—for example, as pollinators selecting for floral traits and mating systems (2, 3) or as herbivores selecting for defense traits (4, 5). Because floral and defense traits are not independent but rather connected through ecological and physiological mechanisms, the effects of pollination and herbivory on plant evolution are also linked to some degree (6–8). For example, whereas plants profit from showy flowers that attract pollinators, such attractive signals sometimes bring in herbivores as well, imposing an ecological trade-off on the evolution of such signals (9, 10). As another mechanism, plant investment in defense, such as the production of toxic metabolites, may compromise pollinator attraction by drawing resources from flower tissues or increasing the toxicity of rewards such as nectar (11), thereby causing physiological trade-offs (12). Nevertheless, pollination and herbivory have traditionally been studied in isolation from each other, and the combined effects of both factors remain a major gap in our understanding of plant evolution (6). Given the commonness of insect pollination and herbivory in angiosperms, it is surprising how little we know about the effects of herbivores on the evolution of pollinator-attracting signals or the combined effects of herbivores and pollinators on the balance between self-pollination and outcrossing (6, 9, 13).

Most studies of plant evolution are limited to hypothesizing or predicting evolutionary trajectories for species and traits, because they either use a comparative approach or study factors relevant for evolution (e.g., selection) within a single plant generation. Experimental evolution under controlled conditions can close this gap by directly demonstrating the (short-term) evolu-

tionary consequences of ecological interactions. Although experimental evolution studies have traditionally focused on organisms with very short generation times, a few recent studies have dealt with plants and interacting insects by using either pollinators (3, 14, 15) or herbivores (4, 5) as selective agents, but no real-time evolution study so far has included the combined effects of both.

For these reasons, mechanisms of plant evolution driven by different interacting organisms are little understood. Our study addresses this topic with experimental evolution using fast-cycling *Brassica rapa* plants and combined pollination by bumble bees and herbivory by caterpillars as selective agents. We studied the evolution of floral traits (i.e., morphology and scent), the mating system (self-compatibility and autonomous selfing), and plant defense (glucosinolates). In our greenhouse experiment, plants from the source population were split into four treatment groups, varying in the type of pollination and the presence or absence of herbivores (*Pieris brassicae* larvae on leaves) (16): (i) hand pollination without herbivory (control); (ii) hand pollination with herbivory; (iii) bee pollination with herbivory; and (iv) bee pollination without herbivory (fig. S1). We replicated each treatment three times with 36 plants each, and each replicate evolved independently during six consecutive plant generations. At generations 7 and 8, all plants were grown without insects to reduce maternal effects caused by herbivory; plants of generation 7 were manually crossed between replicates (but only within the treatment groups) to restore heterozygosity and reduce inbreeding effects (17). Plants were phenotyped to detect consistent evolutionary divergence between treatment groups. In our analysis, we focused on the

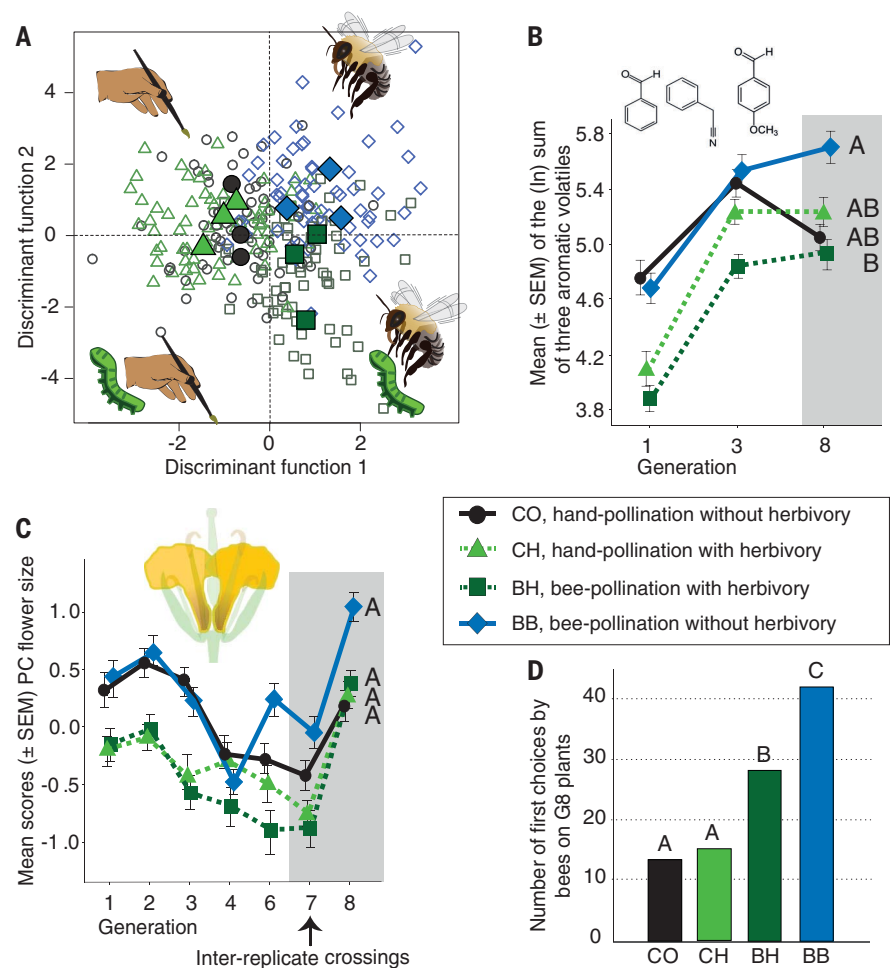
Table 1. The effects of bee pollination, herbivory, and their interaction on the evolution of floral traits, nectar and leaf glucosinolates, and the mating system. Values in parentheses are sample sizes. All traits with a significant effect (indicated by Y for “yes”) of either bee pollination, herbivory, or their interaction are shown (see table S2 for full statistical values). (+) indicates a positive effect (increase) whereas (–) indicates a negative effect (decrease) of either bee pollination or herbivory (see table S1 for exact values). N, no significant effect.

Trait	Bee pollination	Herbivory	Interaction of bee pollination and herbivory
<i>Morphology</i>			
Sepal length (391)	Y(+)	N	Y
Petal width (391)	Y(+)	N	N
Pistil length (391)	N	Y(–)	N
<i>Floral volatiles</i>			
Z-3-Hexenyl acetate (344)	N	N	Y
Phenylacetaldehyde (344)	Y(+)	Y(+)	Y
Benzyl nitrile (344)	Y(+)	Y(+)	Y
p-Anisaldehyde (344)	Y(+)	N	Y
(E,E)- α -Farnesene (344)	N	N	Y
Total volatile emission (344)	N	N	Y
<i>Floral nectar glucosinolates</i>			
Glucanapin (123)	N	N	Y
Glucoraphanin (123)	N	N	Y
<i>Leaf glucosinolates</i>			
Hydroxyglucobrassicin (253)	N	Y(+)	Y
Methoxyglucobrassicin (253)	N	Y(+)	Y
<i>Mating system</i>			
Self-compatibility (384)	N	N	Y
Autonomous selfing (384)	N	N	Y
Herkogamy (391)	N	Y(–)	N

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Fig. 1. Evolutionary changes in floral traits and bee preferences. (A) Multivariate analysis (linear discriminant function analysis; $N = 257$ samples) used to examine differences among replicates of the different treatment groups. Enlarged filled symbols depict the group centroid per replicate. In the analysis, only the replicate, not the treatment group, was predefined. For statistical values, see (16). (B and C) Changes in floral traits during experimental evolution. Trait differences among treatments with and without herbivory in the first generations are due to phenotypic plasticity; the shaded areas indicate generations where insects were no longer applied. Trends toward decreased size common to all treatment groups likely show the effects of inbreeding. (B) Sum of data for three aromatic floral volatiles (benzaldehyde, benzyl nitrile, and *p*-anisaldehyde) that showed a trend or significant increase in the BB treatment group (GLMM; $N = 344$ samples; different letters refer to $P < 0.05$). (C) Principal component (PC) representing petal length and width ($N = 391$ samples). Data for generation 5 were not available. (D) Bioassays testing the preferences (first choices) of bumble bees for plants of different treatment groups at generation 8 (G8). Bees significantly preferred plants that evolved under bee pollination without herbivory (GLMM; $N = 80$ bees and 320 plants; different letters refer to $P < 0.05$). For statistical details, see (16).



following questions: How do floral traits and attractiveness, defense, and mating systems evolve with different pollination and herbivory treatments? Does the interaction between bee pollination and herbivory lead to distinct evolutionary trajectories in experimental plants?

Using a multivariate analysis including 19 floral traits, we showed that plants in our treatment groups differed significantly at generation 8, demonstrating divergent trait evolution during our experiment (Fig. 1A). As expected, bee pollination caused more intensive evolutionary changes in plant traits than leaf herbivory alone, compared with traits in control plants, but the presence of herbivores changed the evolutionary outcome of bee pollination to some degree (Fig. 1A). In more detail, bumble bee-pollinated plants without herbivory evolved more fragrant flowers, which tended to be larger (Fig. 1, B and C, and table S1). Bumble bees clearly preferred those flowers over the flowers of all other treatment groups at generation 8 (Fig. 1D), showing that the plants had adapted to the bees' preferences during the experiment (3). With the addition of herbivores, plants with bumble bee pollination did not evolve a detectable increase in floral signals (Fig. 1, B and C) and the flowers of these plants were less attractive to bumble-

bees (Fig. 1D) than the flowers of plants that evolved with bumble bees only, but they were still more attractive than flowers of plants that evolved with hand pollination (Fig. 1D). These results show that herbivory compromised the evolution of attractive flowers. This was likely caused by resource reallocation, as plants assigned more resources to defense at the cost of investment in reproductive tissues and floral signals, manifesting a physiological trade-off (8, 18). Consistently, we found that defense traits were influenced by the mode of pollination in our experiment. Although only one glucosinolate compound increased in plants with hand pollination and herbivory (table S1), we detected a significant interactive effect of herbivory and bee pollination for two leaf and two nectar glucosinolates (Table 1). Although the physiological link between defense compounds, floral signals, and reward needs more in-depth investigation, we show here that the evolution of both glucosinolate defense and floral attractiveness is influenced by the combined effects of pollination and herbivory.

Besides influencing the evolution of floral traits and plant defense, pollination and herbivory are also key factors determining mating system evolution (13, 19). A common switch in plant

mating systems is the change from primarily outcrossing to self-pollination (20), which is thought to be driven predominantly by pollen limitation (21). Although the combined effects of pollination and herbivory on mating system evolution are little understood, the usually detrimental effects of herbivory on pollinator attraction and therefore pollen income (8, 22, 23) led to the idea that herbivory may indirectly promote selfing or mixed mating through increased pollen limitation (13). We found that herbivory in bee-pollinated plants led to an increase in self-compatibility (Fig. 2, A and B, and tables S3 and S4) and autonomous selfing (Fig. 2, C and D, and tables S3 and S4) compared with these traits in the control group and plants of the first generation. Moreover, we found that both self-compatibility and autonomous selfing were affected by the interaction between bee pollination and herbivory (Table 1). These results may have been caused by a change in bee behavior triggered by herbivore-induced phenotypic changes in flowers. In agreement with earlier studies (24), we showed that bees spent less time on herbivore-infested plants than on plants without herbivory [measured only in generation 1; mean \pm SD, 78.80 \pm 117.09 s and 96.45 \pm 108.07 s; generalized linear mixed model (GLMM),

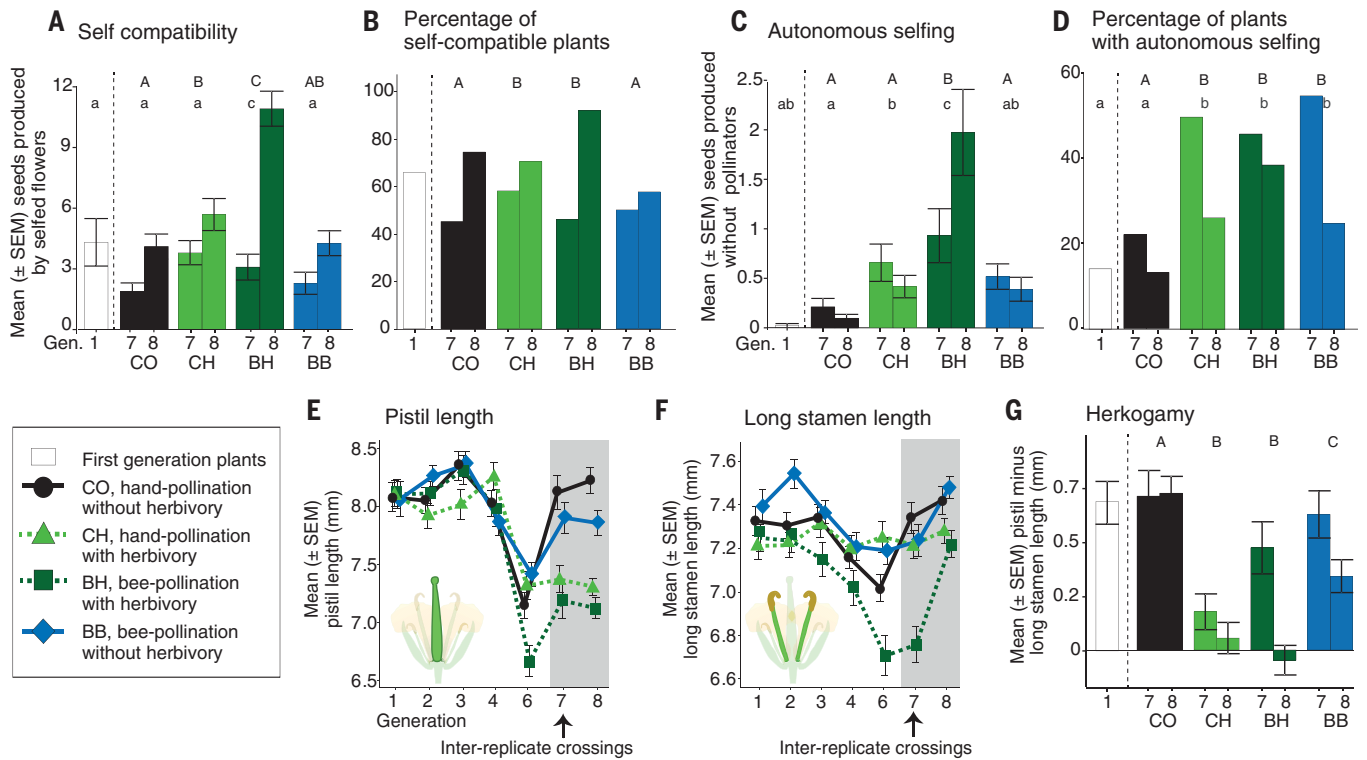


Fig. 2. Evolutionary changes in mating system and flower morphology in experimental plants. (A) Self-compatibility (measured as seeds produced by manually self-pollinated flowers) and (B) the percentage of plants with at least one seed formed after manual self-pollination were higher in bee-pollinated plants with herbivory (BH) (GLMM; $N = 35$ samples for generation 1 and 63 to 91 samples per generation and treatment group for generations 7 and 8; different uppercase letters refer to $P < 0.05$ between treatment groups, and different lowercase letters refer to $P < 0.05$ between plants of generation 1 and treatment groups with generations 7 and 8 pooled). The factor “generation x treatment” was not significant in any GLMM,

except for the percentage of self-compatible plants (see tables S3 and S4 for statistical values). Gen., generation. (C) Autonomous selfing (measured as seeds produced by flowers without access to any pollinators) was elevated in bee-pollinated plants with herbivory (BH) and found with greater frequency in plants of generation 7 or 8 than in plants of generation 1 (D). (E and F) Evolutionary change in pistil (E) and long stamen (F) lengths throughout the experiment (without generation 5). Shaded areas represent plants grown without insects. (G) Herkogamy (the spatial separation of sexual organs in a flower) was reduced in both treatment groups that experienced herbivory (CH and BH) (GLMM, $N = 104$ samples per generation and treatment group).

$N = 101$ samples; estimate = 1.557, z -score = 2.43, $P = 0.015$]. As a consequence, shorter bee visits to herbivore-infested plants may have increased pollen limitation, thus selecting for enhanced selfing (2, 3). In this way, herbivore-induced changes, although a form of phenotypic plasticity and not evolution, can affect evolutionary change by altering pollinator behavior and hence selection on plant traits.

Autonomous selfing depends on self-compatibility and flower morphology that permits self-pollen to be deposited on stigmas (25). One mechanism that modulates the potentially detrimental deposition of self-pollen is herkogamy, the spatial separation between sexual organs in a flower (20). In our starting population, herkogamy was evident in pistils being longer than the long stamen (see illustrations in Fig. 2, E and F). At the end of our experiment, herkogamy remained unchanged in hand-pollinated plants without herbivory, whereas in plants with herbivory (independent of pollination), it evolved to be reduced so that sexual organs overlapped (Fig. 2G and table S3). This overlap of the sexual organs was mediated by a

reduction in pistil length rather than an elongation of the long stamen (Fig. 2, E and F).

Reduced herkogamy likely facilitated autonomous selfing in our experimental plants and may have evolved under selection for reproductive assurance (2). We found negative selection on herkogamy in plants with bee pollination and herbivory (table S5), suggesting pollen limitation as a selective factor. On the other hand, plants with hand pollination and herbivory also evolved reduced herkogamy but not via direct selection, because in these plants the degree of pollen limitation was obviously not influenced by the presence or absence of herbivory. Alternatively, reduced herkogamy may be modulated by pleiotropy or genetic correlation with signaling by jasmonate, a plant hormone involved both in defense responses against herbivores and in flower development (26, 27). Such a physiological link between defense and traits involved in mating systems may be adaptive, especially in annual plants, where the time available for outcrossing is limited and fitness under herbivore attack is optimized with selfing.

In conclusion, we have demonstrated rapid evolutionary changes in plants driven by mutualists and antagonists. We found that nonadditive interactions between pollinators and herbivores shape the evolution of the majority of traits with very different yet physiologically and ecologically linked functions, such as pollinator attraction, defense, and mating (Table 1). The importance of such interactive effects points out the need for more integrative studies to better understand how interactions shape the evolution of diversity. Understanding mechanisms of evolution has never been more relevant as in contemporary times, where human-altered environments change evolutionary fates of a multitude of organisms, with consequences for ecosystem functioning, biodiversity loss, and food security (28).

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/364/6436/193/suppl/DC1
Materials and Methods
Figs. S1 and S2
Tables S1 to S7
References (29–33)

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Adaptation by way of compromise

Many plants rely on animal pollinators to spread pollen and increase the genetic diversity of their offspring. However, there are trade-offs, because attracting pollinators may also attract herbivores and deterring predation may diminish floral displays. Ramos and Schiestl studied the interplay between mating system, flowers, and chemical defenses over several generations in *Brassica rapa* plants (see the Perspective by Ågren). Evolution driven by pollination and herbivory can be observed after only eight generations, suggesting that trade-offs have large evolutionary consequences.

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