predicted by the COPSE model (*16*). This model, unlike GEOCARBSULF (*2*) and the model by Falkowski *et al*. (*3*), includes fire feedbacks and nutrient cycling and accounts for their controls on terrestrial productivity. These are known to affect weathering and organic carbon burial rates (*18*), which in turn play a role in regulating O_2. Future models of paleoatmospheric O_2 should strongly consider the effects of changing terrestrial and marine productivity, including fire feedbacks.

We have shown that extensive periods of low O_2 (<15%) cannot have occurred in the Mesozoic, according to our revised lower limit for combustion coupled with the record of paleowildfires. This also suggests that the predicted low O_2 (<13%) levels for the Frasnian (385 to 374 Ma) in the Palaeozoic (*19*, *20*) needs reevaluation. The paleowildfire record provides a key means for testing low-O_2 events in the geological record and highlights the need for high-resolution studies of paleowildfire across major mass-extinction events in order to test current hypotheses that advocate a primary role of short-term low-atmospheric-O_2 events in catastrophic faunal diversity loss in the Permian-Triassic (*4*) and Triassic-Jurassic (*21*) mass-extinction events.

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Field Experiments with Transformed Plants Reveal the Sense of Floral Scents

Danny Kessler, Klaus Gase, Ian T. Baldwin*

Plants use many means to attract pollinators, including visual cues and odor. We investigated how nonpigment floral chemistry influences nectar removal, floral visitation, florivory, rates of outcrossing, and fitness through both male and female functions. We blocked expression of biosynthetic genes of the dominant floral attractant [benzyl acetone (*Nachal1*)] and nectar repellent [nicotine (*Napmt1/2*)] in all combinations in the native tobacco *Nicotiana attenuata* and measured their effects on plants in their native habitat. Both repellent and attractant were required to maximize capsule production and seed siring in emasculated flowers and flower visitation by native pollinators, whereas nicotine reduced florivory and nectar robbing.

Flowers produce bouquets of scents that are believed to help reproduction by attracting pollinators. However, experimental proof that floral scents facilitate outcrossing is lacking. In contrast, the effects of visual traits on pollination have been well studied (*1*), and floral pigments clearly influence pollination (*2*, *3*). The ability to manipulate nonpigment floral chemistry—to evaluate its importance in attracting floral visitors, nectaring times, and thus, mediate outcrossing—has been lacking. Floral scents are generally believed to function by tuning the largely visual process of attracting pollinators (*1*). Moreover, attracting pollinators may increase the probability that a plant will draw herbivores (*4*, *5*), and thus, fragrance bouquets may also be defensive (*6*).

Department of Molecular Ecology, Max-Planck-Institute for Chemical Ecology, Hans-Knöll-Strasse 8, DE-07745 Jena, Germany.

*To whom correspondence should be addressed. E-mail: baldwin@ice.mpg.de

Flowers face a multidimensional challenge because they need to attract visitors, compel them to vector pollen with the least investment in rewards, and repel nectar thieves, robbers, and florivores (*7*, *8*). Previous work with native floral visitors of the white-flowered wild tobacco plant *Nicotiana attenuata* demonstrated that nicotine (N) and benzyl acetone (BA) are the most abundant repellent and attractant, respectively, of 16 floral secondary metabolites (*9*). Experiments with artificial flowers demonstrated that hummingbirds and hawkmoths removed less nectar but increased their visits if the nectar contained N. BA, which is released from the outer lips of the corolla at night (*10*), was consistently attractive to all flower visitors, increasing their time spent removing nectar (*9*). The BA synthesis pathway in flowers is generally unknown but may involve a polyketide synthase functioning as a chalcone synthase (*11*). We identified two plasmids (pFLO10 and pFLO70) in a cDNA library of corolla tissues (*12*) carrying overlapping inserts (CF920188 and FE192200) with 99% identity to each other and 86% identity to the 5′ end of the *Nicotiana tabacum* chalcone synthase mRNA (AF311783). These sequences were used to clone fragments of *Nachal1* (EU503226), which is expressed in both leaves and flowers, and *Nachal2* (EU503227), which is expressed only in the leaves (*12*). *Nachal1* transcripts in corollas varied diurnally, attaining maximum levels 4 hours before the crepuscular peak of BA emission (fig. S1D) (*12*). Transgenic plants with RNA interference (RNAi) constructs harboring fragments of endogenous biosynthetic genes in an inverted-repeat orientation were used to block BA (*Nachal1*), N [putrescine N-methyltransferases (*Napmt1/2*)], and both BA and N (*Nachal1*/*Napmt1/2*). Silencing the expression of *N. attenuata*'s *Napm* (1/2) genes dramatically reduces N accumulation throughout the plant (*13*), including its nectar (*9*).

The RNAi constructs only blocked the targeted biosynthetic pathways, and the transformed plants were morphologically indistinguishable from empty-vector transformed and wild-type (WT) plants of the same inbred generation that was used for the transformations. Nectar volume, sugar concentration, and floral and vegetative volatiles (control and herbivore-induced) were also unchanged (fig. S4, B and C, and table S2). T_3 generation lines, each homozygous for a single transgene insertion with diminished levels of BA, N, or both BA and N accumulations, were used for all experiments (fig. S1B) (*12*). We arrayed transformed and WT individuals in an irrigated field plot (fig. S1D) at the Lytle Ranch Preserve (*12*).

To protect native populations of *N. attenuata* from unwanted escape of the transgenes, all plants were monitored daily, and all flowers of each

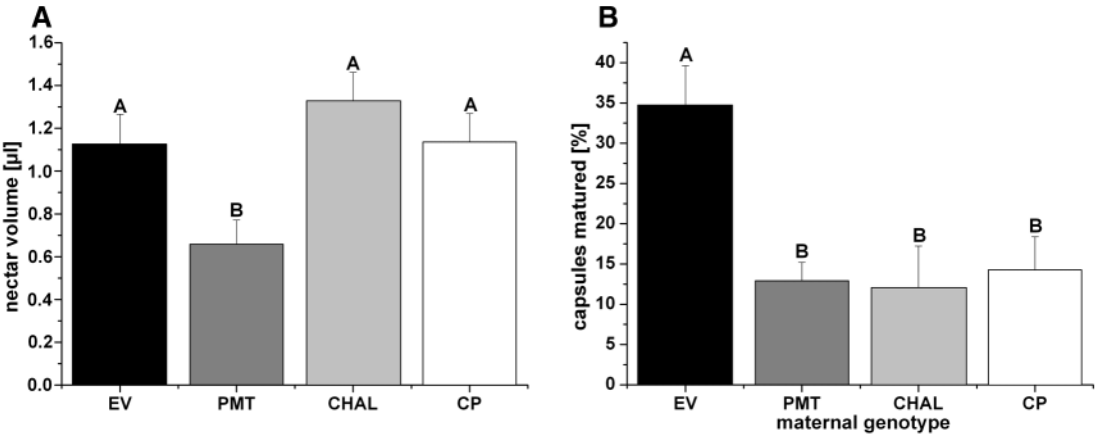

Fig. 1. Nectar removal by floral visitors and capsule production resulting from outcrossing mediated by pollinators in EV *N. attenuata* plants and plants transformed to block BA, N, or both in the plant's native habitat. (**A**) Mean (±SE) standing nectar volume between 6:00 and 7:30 a.m. in flowers that had opened for the first time the previous night [see fig. S4D for the individual day mean (±SE) values]. (**B**) Mean (±SE) percentage of capsules matured from 601 antherectomized flowers from each genotype (see fig. S8A for values from each day). Different letters (A and B) designate significantly different means as determined by a Fisher's protected least significant differences (PLSD) test ($P < 0.05$) of an ANOVA.

transformed plant not explicitly part of an experiment were removed before they released any pollen. Experimental flowers were labeled and emasculated (fig. S6) (*12*) or used as pollen donors. Each mature capsule growing in the field plot was collected before any seeds were released, and all material was destroyed upon completion of the experiment.

To determine whether the genotypes differed in nectar accumulation and whether native floral visitors preferred a particular genotype's nectar, nectar volume was measured between 5 and 7 a.m., when most nectar accumulates (fig. S4, A and B) (*12*). Nectar was measured from flowers of field-grown plants exposed to floral visitors, field-grown plants enclosed in mesh bags that excluded floral visitors but allowed for evapotranspiration, and greenhouse-grown plants not exposed to floral visitors (fig. S4C). No differences in nectar volume were found among the genotypes in greenhouse- and field-grown plants not exposed to floral visitors [analysis of variance (ANOVA) $F_{3, 58} = 1.44$, $P = 0.242$; $F_{3, 33} = 0.15$, $P = 0.930$] (fig. S4, B and C) (*12*). However, field-grown plants lacking N that were exposed to flower visitors had significantly less nectar volume than flowers producing N (Fig. 1A), confirming that N functions as a deterrent and that its absence increases consumption of nectar (*9*). In exposed plants, nectar volumes in plants lacking both N and BA did not differ from those transformed with an empty vector, suggesting that blocking BA emissions reduced pollinator visitation (Fig. 1A).

We video monitored the activity of floral visitors (fig. S11B) (*12*) and saw that plants lacking BA (both with or without N) received fewer visits from hawkmoths and hummingbirds (fig. S12, A and B) than those with BA-producing flowers. Hummingbirds and hawkmoths spent the least time nectaring (estimated from beak- and proboscis-insertion times) flowers that lacked N [carrying transferred DNA (T-DNA) of pRESC5PMT (PMT) or pRESC1CHAL and RESC5PMT (CP)] as compared with flowers containing N [carrying

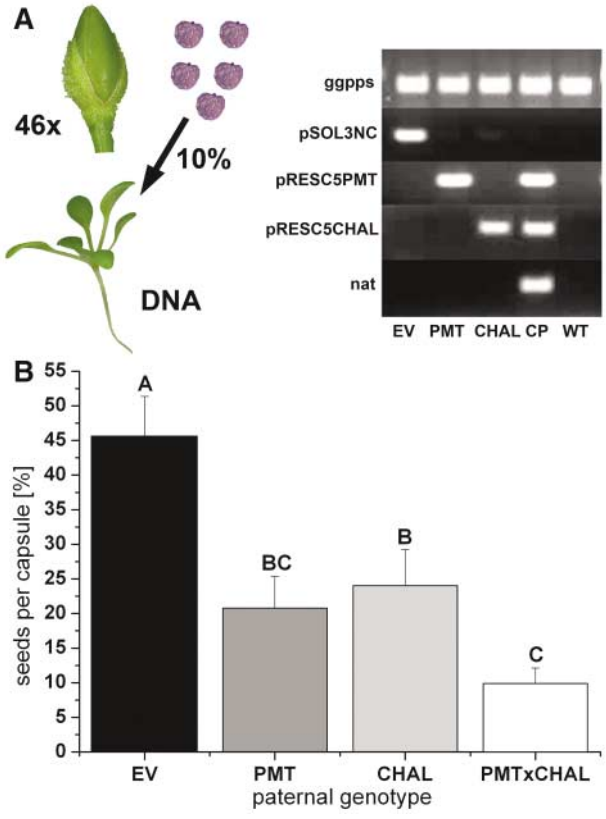

Fig. 2. Paternity of seeds sired by cross-pollination of antherectomized WT flowers. (**A**) From each of the 46 capsules that matured seeds (fig. S8A), 10 or 10% of all seeds were germinated (*13*). DNA was extracted from the 2-week-old seedlings and analyzed for paternity by polymerase chain reaction to determine seed paternity. ggpps, *N attenuata* geranylgeranyl diphosphate synthase gene (EF382626); pSOL3NC, empty vector control; pRESC5PMT, *Napmt 1/2* RNAi construct; pRESC5CHAL, *Nachal 1* RNAi construct; nat, nourseothricin resistance gene *Sat-1* (X15995). (**B**) Mean (±SE) percentage of seeds per capsule sired by one of the four genotypes. Different letters (A, B, and C) designate significantly different means as determined by a Fisher's PLSD test ($P < 0.05$) of an ANOVA.

T-DNA of pSOL3NC(EV) or pRESC5CHAL], both with and without BA. This was particularly true of hummingbirds (figs. S12A and S11C). These results highlight the influence of BA emissions and nectar N on flower visitors, but whether these secondary metabolites increased plant fitness by increasing outcrossing rates was not clear.

Because *N. attenuata* is a fully self-compatible but opportunistic outcrossing species (*14*), we removed the anthers to measure outcrossing rates (*12*). Antherectomies were performed typically 12 hours before floral anthesis (fig. S6) (*12*). To determine the efficiency of the antherectomy in preventing self-pollination and to measure the rate of cross-pollination in natural populations of *N. attenuata*, we antherectomized two flowers on each of 44 WT *N. attenuata* plants from a native population, 19 km away from our experiment, and removed all other flowers and buds. Half of these plants were then covered with a mesh-wrapped wire cone (fig. S7A) that excluded all flower visitors, and the other half was covered after 36 hours. None of the covered plants matured capsules from antherectomized flowers, whereas plants exposed to flower visitors had 90% (28/31) of the antherectomized flowers mature.

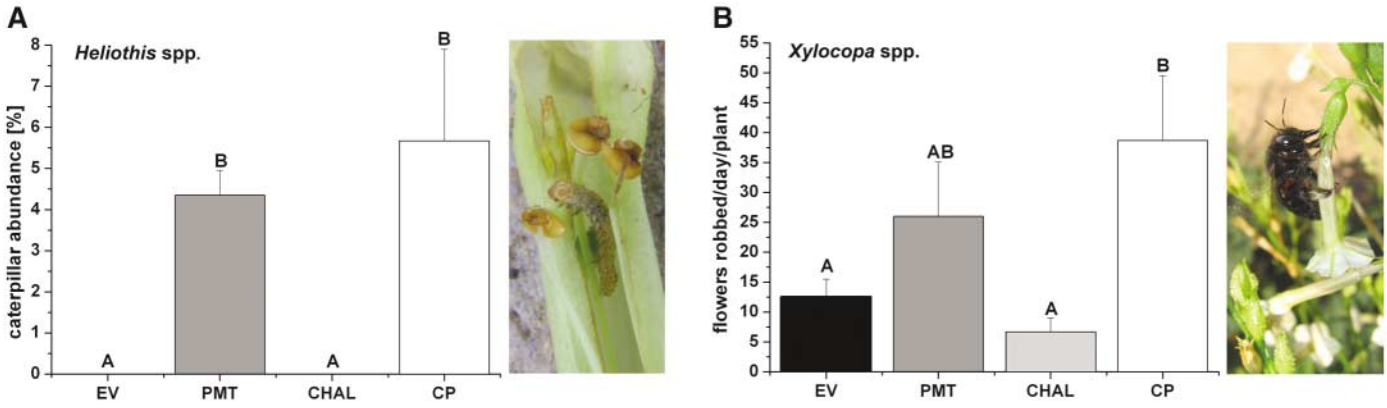

Fig. 3. (**A**) Mean (±SE) percentage of flowers damaged by tobacco budworm (*Heliothis* spp.) larvae. (**B**) Mean (±SE) flowers robbed by carpenter bees (*Xylocopa* spp.) per day and plant. Different letters (A and B) designate significantly different means as determined by a Fisher's PLSD test ($P < 0.05$) of an ANOVA.

We found no differences in pollen number, fertility, or ability to competitively sire seeds among the transgenic genotypes and WT plants (*12*). Therefore, differences in the maternal and paternal fitness among the genotypes in the field plantation could be attributed to the ability of flowers to attract pollinators, remove pollen, and transport pollen to receptive stigmas. To measure the effects of floral BA and N on female fitness, we emasculated three flowers on 41 to 60 plants of the transgenic genotypes on five days during the growing season; all other flowers were removed from these plants on these days. Flowering WT plants growing around the perimeter of the field plot (fig. S1D) were the only available pollen donors on these dates. In total, 601 flowers were emasculated. During one particularly windy day, no pollinators were active and no capsules were produced from the 127 emasculated flowers. A total of 87 mature capsules were produced from the 474 antherectomized flowers on the remaining 4 experimental days (fig. S8A); 45 of these were lost at an early stage of development as a result of herbivore feeding or plant death. Capsule production averaged across the 4 experimental days (12 to 14%) was significantly lower in PMT, CHAL, and CP plants as compared with EV plants (35%) (Fig. 1B and fig. S8A), demonstrating that plants' ability to attract pollinators and cross-pollinate with WT flowers was lower if their flowers lacked BA, N, or both. The number of seeds produced per capsule did not significantly differ among genotypes [ANOVA $F_{3,61} = 0.170$, $P = 0.92$; CHAL, 115 ± 21 (mean ± SE); PMT, 117 ± 43; CP, 96 ± 19; EV, 108 ± 17], showing that pollinator visits to individual flowers were the limiting factor in an individual's fitness.

To measure the effects of floral BA and N on male fitness, we antherectomized and labeled between one and eight flowers on 19 to 24 WT plants on 5 days during the growing season, totaling 502 flowers. All other WT flowers were removed. To ensure that each of the EV, PMT, CHAL, and CP genotypes had the same opportunity to function as pollen donors for the emasculated WT flowers, we manipulated the plants to result in the same number of open flowers for all genotypes (fig. S9B). Matured capsules were collected before opening, seeds were counted, and paternity was determined (Fig. 2A) (*12*). Across all 5 experimental days, EV plants sired 1.9 times more seeds in emasculated WT flowers than did CHAL plants, 2.2 times more seeds than PMT plants, and 4.7 times more seeds than CP plants (Fig. 2B and table S4). Although EV pollen was uniformly better than genotypes lacking N or BA, over the season the success of CHAL pollen tended to decrease (Student's t test, $t_{23} = 2.15$, $P = 0.043$), whereas PMT pollen success tended to increase (Student's t test, $t_{23} = -2.16$, $P = 0.042$) (fig. S9A), suggesting that the effects on male fitness wane for N and increase for BA over time.

These changes in male and female function as a result of changes in N and/or BA levels correlated with the relative frequency of visits from the main floral visitors as observed on video. Hummingbirds were more frequent floral visitors early in the growing season, and hawkmoths were more frequent later (fig. S11A). This correlation between plants' fitness through the male function and the activity of the floral visitors is consistent with earlier observations that hummingbirds respond strongly to the presence of N in nectar (*9*), which increases outcrossing rates. In short, the lower fitness of N- and BA-blocked flowers reflected the distinct functions of these secondary metabolites: BA is correlated with increased pollinator visits, whereas N appears to enforce modest drinking behavior.

The daily manual inspection and video monitoring also provided quantitative data on florivory and nectar robbing. *Heliothis* spp. larvae were found feeding within the closed flowers at the stage in which flowers were emasculated (Fig. 3A) in 4.3% of PMT and 5.2% of CP flowers but in no WT flowers (of 638 emasculated flowers). Nectar-robbing carpenter bees (*Xylocopa* spp.) showed the same preference for N-deficient flowers, although attacks were higher on CP plants than PMT plants (Fig. 3B), suggesting that BA synergizes the defensive effects of N in mediating resistance to this nectar robber. On the basis of these data, we conclude that combinations of repellents and attractants help flowers avoid predators while attracting mates. Unlike animals, plants are sessile, but through chemistry, flowers can optimize visitors' behavior.

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Supporting Online Material

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Danny Kessler *et al.*

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