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Opportunistic out-crossing in *Nicotiana attenuata* (Solanaceae), a predominantly self-fertilizing native tobacco

Karen R Sime^{1,2} and Ian T Baldwin*¹

Address: ¹Department of Molecular Ecology, Max-Planck-Institute for Chemical Ecology, Jena 07745, Germany and ²Current address: Division of Insect Biology, University of California, Berkeley, California 94720 USA

Email: Karen R Sime - ksime@nature.berkeley.edu; Ian T Baldwin* - baldwin@ice.mpg.de

* Corresponding author

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Abstract

Background: Although *Nicotiana attenuata* is entirely self-compatible, chemical and other floral traits suggest selection for the maintenance of advertisement for moth pollinators.

Results: Experimental exclusions of pollinators from plants with emasculated flowers in natural populations in southern Utah during an outbreak of the hawkmoth *Hyles lineata* revealed that 24% of the seed set could be attributed to insect pollination, and eliminated wind pollination and apomixis as contributing to seed set. Hence these moths can mediate gene flow when self-pollen is unavailable. To quantify gene flow when self-pollen is available, plants were transformed with two marker genes: hygromycin-B resistance and β -glucuronidase. The utility of these genetic markers to measure gene flow between plants was examined by mixing pollen from plants homozygous for both genes with self-pollen in different ratios and hand-pollinating emasculated flowers of plants growing in a natural population. The proportion of transformed seeds was positively correlated with the amount of transformed pollen applied to stigmas. In glasshouse experiments with the hawkmoth *Manduca sexta* and experimental arrays of transformed and wild-type plants, pollination mediated by moths accounted for 2.5% of the seed set.

Conclusions: Even though moth pollination is rare and highly variable for this largely selfing plant, *N. attenuata* opportunistically employs a mixed-mating system.

Background

The wild tobacco *Nicotiana attenuata* Torrey ex Watson (Solanaceae) represents a model system in chemical ecology and is of particular importance because it has been subject to intensive study in its natural habitat, the Great Basin deserts of North America, as well as under laboratory conditions. Investigations of germination cues, induced resistance to insect herbivores, interactions with the third trophic level, and competitive interactions and

costs of defense have provided a remarkably detailed picture of the adaptations of this annual species to its environment [1–6]. The picture cannot be complete, however, without a thorough understanding of its reproductive system and strategies.

Nicotiana attenuata is an ephemeral, early-successional species. Large populations, which may consist of tens of thousands of individuals densely spread over several

hundred hectares, occur for 1 to 3 years after fires in sage-juniper habitats, and much smaller populations often persist in roadsides, dry washes, and other disturbed areas [5,7,8]. Between fires – the intervals average 10 to 30 years and are often greater than a century – the *N. attenuata* populations survive as a dormant seed bank that germinates in response to smoke-related cues [5]. The plants produce white tubular flowers that open after sunset. Each flower is open for two or three consecutive nights, and each plant may produce hundreds of flowers over several weeks. Anthesis usually occurs shortly before the flowers open on the first night, and most seeds set in nature are thought to result from self-pollination. Field experiments have revealed no decrease in seed set or viability from flowers bagged to exclude pollinators compared to uncovered controls, confirming that the species is fully self-compatible ([9]; IT Baldwin, unpublished results). Moreover the lack of any apparent inbreeding depression after more than 20 consecutive generations of selfing of six field-collected genotypes (IT Baldwin unpublished results) is consistent with recent population bottlenecks and/or a history of selfing.

Nonetheless, *N. attenuata* has many features that are consistent with the maintenance of outcrossing. The color and shape of the flowers is typical of moth-pollinated plants [10]. Each flower produces much more pollen than necessary: five fully-developed anthers are present, but full seed set occurs after hand-pollination of emasculated flowers with the pollen equivalent of just one anther [9]. The flowers exhibit variation in the relative position of stigma and anthers (unpublished data) [11]. The emission of floral volatiles increases dramatically in the evening and decreases abruptly the day after (hand) pollination [12]. Not only is this pattern of odor emission typical of moth-pollinated plants [13], but it is costly to *N. attenuata* because it attracts herbivores and seed predators [9]. That such features persist despite risks suggests that they provide an advantage to the plant and are not simply ancestral relics.

In particular, benzyl acetone, the main component of the floral odor of *N. attenuata* [12], is structurally similar to two compounds, amyl salicylate and amyl benzoate, known to attract adult *Manduca sexta* (L.) (Sphingidae) [14]. The larvae of *M. sexta* and the closely related *M. quinquemaculata* (Haworth) feed on *N. attenuata* and inflict significant damage [3,4,15], which implies that advertisement to the adults, who lay eggs at night, is risky. It is not obvious, though, that the flowers and the moths are well matched: the flowers' standing pool of nectar during maximum floral emission (typically 1–3 μ l) is small compared to that of other species visited in the habitats (IT Baldwin unpublished results), and the moths' tongues, at 10–12 cm [16], are much longer than the *N. attenuata*

corolla (about 3 cm [17]), so that only the tongue potentially comes in contact with the anthers. Nonetheless, the flowers are apparently attractive and the moths occasionally visit them in the field ([18], IT Baldwin unpublished results).

Here we investigate the ecological context of the floral features of *N. attenuata* by determining whether moth visits result in cross-pollination for this largely selfing species. The 2001 season proved to be exceptional for field observations of pollinator visitation in southern Utah due to an outbreak of another hawkmoth, *Hyles lineata* (Fabricius). This species is potentially a more effective pollinator than the *Manduca* species because the length of its tongue is comparable to the dimensions of the *N. attenuata* corolla [16]. However, although it is widespread in *N. attenuata* habitats, *H. lineata* is not always present. More visits were seen during an average evening in 2001 than had been observed during the previous 16 years of field work in similar populations.

Taking advantage of the outbreak, we performed a series of field studies, including experiments in which we emasculated flowers on plants with and without netting to exclude pollinators and measured seed set. Second, using plants transformed with two readily measurable genetic markers, we measured the competitive ability of transformed pollen with native pollen in natural populations and, in glasshouse arenas, the ability of adult *Manduca sexta* moths to transfer pollen between plants. We conclude that *N. attenuata* faces unpredictable pollinator abundance but can take advantage of whatever visits occur, thus defying classification as selfing or mixed-mating at the species level.

Results

Field investigation of pollen transfer

About 24% of exposed, emasculated flowers set seed (Table 1). That no bagged, emasculated flowers set seed indicates that wind pollination and apomixis do not contribute to seed set and indicates that insects are the most likely vectors of pollen. The emasculated but otherwise untreated flowers and the hand-pollinated, emasculated flowers produced significantly fewer seeds per capsule than uncut flowers (unpaired 2-tailed t-test, $p = 0.0175$ and $p = 0.029$ respectively), though the proportions of viable seeds did not differ. The three treatments in which seeds were produced did not differ from each other in number of seeds per capsule. This experiment did not permit distinction between differences due to acceptance of exogenous pollen, amount of exogenous pollen, or effects from emasculation. No significant effects due to bagging were detected. In all treatments, the seed counts per capsule were lower than the average reported for undisturbed *N. attenuata*, which may perhaps be explained by the

Table 1: Results of a field experiment in which plants were emasculated and exposed, with and without hand pollination with and without enclosure in mesh sleeves (na = not applicable). For the "Untreated" specimens, mature seed capsules were selected from intact, undisturbed plants not included in the experiment; seed set was not recorded.

	Emasculated	Emasculated & hand-pollinated	Emasculated & bagged	Emasculated, hand-pollinated & bagged	Untreated
Total number of flowers	206	37	34	23	(20)
Number setting seed	50 (24%)	14 (38%)	0	4 (17%)	(20)
Average number of seeds per capsule (SE)	68 ± 5	63 ± 8	na	44 ± 25	87 ± 6
Average proportion of viable seeds	0.65	0.68	na	0.77	0.77

Table 2: Mixtures of transformed and wild-type pollen were applied to the stigmas of emasculated flowers at the Hwy 91 field site. In the first experiment (using DI pollen), there was no significant relationship between the concentration of transformed pollen and the number of GUS-positive seedlings. This result may reflect the low rate of seed set and thus small sample size. In the second experiment (Hwy 91 pollen), there was a significant positive relationship (Spearman Rank Correlation: $r_s = 0.975$, $p < 0.05$).

Treatment	Number of plants that set seed	Average number of viable seeds per capsule (± s.e.)	Average number of GUS + seedlings per capsule (± s.e.)
Pollen from DI site mixed with transformed pollen (n = 25 replicate plants)			
100% DI	3	24.3 ± 10.0	0
DI: tr 9:1	6	24.2 ± 16.1	0.17 ± 0.37
DI: tr 1:1	3	82 ± 20	5.0 ± 4.5
DI: tr 1:9	6	12.3 ± 13.2	1.5 ± 1.8
100% transformed	3	20 ± 14.8	1.0 ± 1.7
Pollen from Hwy 91 site mixed with transformed pollen (n = 20 replicate plants)			
100% 91	9	43.2 ± 23.6	0
91: tr 9:1	4	29.3 ± 26.8	1.3 ± 1.3
91: tr 1:1	9	50 ± 33.6	3.1 ± 3.2
91: tr 1:9	11	48.1 ± 33.2	3.1 ± 4.0
100% transformed	9	17.3 ± 17.0	4.1 ± 5.4

relatively high numbers of seed predators (Thyreocoridae: *Cormelina* spp.) observed in 2001 [9]. The low rates of seed set in this and subsequent experiments is most likely a result of the damage done to the flower during emasculation, though this was not explicitly tested. In the pollen mixing experiment, the number of transformed offspring in each seed capsule increased with the increasing concentration transformed pollen applied to the stigma (Table 2). The ratios of transformed seedlings did not correspond to the mixing ratios, being lower than expected if no competition had occurred. However, the emasculation experiments described above showed that extra pollen may have been delivered by pollinators, which would skew the actual ratios of applied pollen towards wild type. The experimental design therefore does not permit any quantitative conclusions about competition but only the conclusion that there is a positive correlation between the amount of exogenous pollen delivered and its representation in the offspring.

White-lined sphinx moths were by far the most common visitors at *N. attenuata* flowers during 160 min of timed observations made at dusk. Hummingbirds and nectar-robbing carpenter bees were occasionally seen as well but tended to visit earlier, while there was more light, and before most flowers had completely opened. Adult *H. lineata* accounted for 146 visits, while only two *Manduca* moths (either *M. sexta* or *M. quinquemaculata*) visited the plants along with a few individuals of two or three unidentified species of smaller moths (Table 3). Like *Manduca* spp., *H. lineata* feed while hovering. Usually only the proboscis contacted the flowers, but the tongue is relatively short (about 3.5 cm [16]) and occasionally other parts of the face touched the stigma. Most visits involved neighboring plants: the average distance traveled between plants was 1.3 ± 0.2 m and the average number of plants visited within a patch before the moth was lost to sight was 6.1 ± 0.7 (range 1–22). The average number of flowers each moth visited on each plant was 2.0 ± 0.1 (range 1–8).

Table 3: Adult *H. lineata* were observed in 5 × 5 m plots within *N. attenuata* patches for 10-minute intervals. The moths' movement patterns were relatively similar and averaged 5.1 plants visited per plot ($r^2 = 0.63$).

Date	Plots observed	Plants in plots (Mean ± SE)	Moths entering plots (Mean ± SE)	Plants visited (Mean ± SE)	Plants visited per moth (Mean ± SE)	% of plants that were visited (without repeats)
July 1	3	90 ± 10	6.7 ± 0.67	25 ± 6.2	4.0 ± 1.2	27
July 2	2	100 ± 20	9.5 ± 0.50	56 ± 3.5	5.8 ± 0.06	82
July 8	3	90 ± 5.7	5.3 ± 2.8	38 ± 15	8.9 ± 2.8	41
July 9	2	90 ± 10	6 ± 0	56 ± 2.5	9.4 ± 0.42	64
July 11	6	130 ± 13	13 ± 2.0	79 ± 12	6.0 ± 0.30	64

On average 51% of plants in a 5 × 5 plot were visited by white-lined sphinx moths in a 10-minute interval (Table 3). The method overestimates visitation rates because repeat visits by different moths were counted as separate plant visits. For example, in plot 14, in which more visits occurred than there were plants, some plants were probably not visited at all. However, this method also might underestimate the probability of visitation. The 51% figure simply covers a 10-minute interval of high moth activity, of which there are five or six on most evenings. The moths are also occasionally active throughout the night. They were observed in high numbers from mid-June to late August, and most plants produce flowers over a period of 2–3 weeks. It thus appears that the chances of any given plant receiving a visit from a moth in its lifetime approached 100% in 2001.

Laboratory investigation of pollen transfer by *Manduca sexta*

In the course of four 15-minute intervals, each of the 12 wild-type plants in the first array was visited at least once; 42 total visits were recorded. Of 40 hand-pollinated flowers, 25 produced seed capsules, and 15 of these capsules included transformed offspring (Table 4). Because the flowers were effectively saturated with visits, and because no positional biases were observed in the distribution of plants producing transformed offspring, the wild-type plants in the array can be considered independent replicates. The capsules averaged 2.1% transformed offspring. In the first array, 9 of the 12 wild-type plants produced seed capsules, and of these, 7 had transformed offspring. In contrast, none of the 8 wild-type plants in the control (third) array produced transformed offspring. The difference ($p = 0.082$, chi-square test for independence) provides strong evidence that moths moved transformed pollen in the first array. The proportions of transformed offspring from the hand-pollinated flowers (2.1%) and from the flowers in the second array (2.3%) did not differ significantly from those in the first array, which indicates that the moths did not accumulate pollen on their pro-

boscus proportionately to the number of transformed plants visited.

Discussion

Hawkmoths are considered important, and in some cases obligate, pollinators for plants in many families [10]. There are relatively few direct, experimental demonstrations of their roles in pollen transfer, however, many of the relationships having been deduced from observations of visits and from examination of pollen on the insects' mouthparts (as reviewed in [13,16]). Experimental studies of species of Solanaceae and Convolvulaceae have demonstrated that hawkmoths are involved in pollination of predominant selfers [19–21]. The GUS and antibiotic-resistance marker genes, which have already found use in pollination biology for field studies of gene dispersal [22–24] and to examine pollen-tube growth [25], provide an additional tool for such investigations, permitting confirmation of the acceptance of exogenous pollen and evaluation of the relative contributions of pollinators to seed set.

The manner in which *Manduca* species handle flowers in the field is not different from their behavior in cages, and it may be assumed that their visits result in a small amount of outcrossing. It was presumably the activities of *H. lineata*, however, that accounted for the relatively high rate of seed set in emasculated flowers in the field experiment described here. No other insects visited the flowers in sufficient numbers. It must be emphasized that the 24% figure does not correspond to an outcrossing rate because it may not reflect the responses of intact flowers, where the timing of the pollen delivery with respect to anthesis and the relative quantities of exogenous and self-pollen may be important. This number simply shows the proportion of flowers that acquired from pollinators the necessary amount of pollen to set seed – the equivalent of one anther.

Because an anther-equivalent represents a considerable quantity of pollen, this figure could underestimate the

Table 4: Marker genes were used to trace pollen transfer from transformed to wild-type plants with intact anthers. A hand-pollination test was used to evaluate the effect of direct transfer on seed set. Moths (*M. sexta*) visited two arrays (1 and 2) of wild-type and transformed plants but were excluded from the third array.

Treatment	Wild-type plants in treatment	Wild-type plants setting seed	Plants with transformed offspring	Seeds per capsule (Mean ± SE)	Transformed offspring per capsule (Mean ± SE)
Hand-pollination	8	8	8	154.2 ± 8.0	3.2 ± 1.3
Array 1	12	9	7	124.6 ± 11.8	2.4 ± 0.9
Array 2	1	1	1	148.5 ± 15.2	3.5 ± 1.5
Array 3	8	7	0	105.6 ± 7.2	0

number of flowers that received some pollen. It is reasonable to conclude that bursts of outcrossing are possible in outbreak years, though we cannot precisely quantify the outcrossing rate in this study. Schemske and Lande [26] classified as selfers plants with <20% outcrossing rates, while plants with 20–80% were considered to have mixed mating systems. Others have argued that stable mixed mating systems can involve less than 10% and often less than 1% outcrossing [20,27]. Neither category precisely describes *N. attenuata*, for the outcrossing rates of populations are likely to vary greatly. The high rate of pollen transfer attributed to *H. lineata* in 2001 will seldom be observed: *H. lineata* is considered an irregular outbreak species in the Great Basin deserts [28,29] and was rare in *N. attenuata* habitats for the 16 years preceding the current outbreak (IT Baldwin personal observations). If, for example, a comparable outbreak happens every 20 years, and if a population of *N. attenuata* appears every 50 years, then the chances of seeing a similar result in a population would be one in 1000. Other moths are consistently present in smaller numbers, and in any year will transfer some pollen. The observations in 2001 suggest that *Manduca* and other moths would visit only a fraction of one per cent of plants, and this percentage probably better represents the outcrossing rate within most *N. attenuata* populations.

If sporadic bursts of outcrossing have occurred consistently over evolutionary time for the *N. attenuata* populations in Utah, then they would be expected to have heterozygosity measures that are higher than those typically found in other predominantly selfing annual plants [30]. A recently completed AFLP and ISSR analysis of 244 individuals collected from populations growing in 11 wash and burns over 11 years from Utah (Bahulikar *et al.* in review) revealed that total heterozygosity measured from all populations (0.203), was higher than the average (0.131) reported from comparable analyses of self-pollinating annual plants [30]. Hence the genetic signatures expected from sporadically high outcrossing are found in these populations.

Variable outcrossing rates are probably common among sphingid-pollinated plants. Hawkmoths are not considered to be particularly reliable pollinators because populations vary from year to year and flight activity depends on environmental conditions [10]. Many sphingophilous plants, including other *Nicotiana* species, therefore have backup mechanisms to insure or at least increase the likelihood of pollination. These include self-compatibility, the production of large numbers of flowers, relative longevity of plants and flowers compared to moth flight periods, and the production of many seeds from each flower. All of these features are found in *N. attenuata*.

This study only considered pollen transfer within populations. Because their movements were fairly localized, most pollen transfer resulting from the visits of *H. lineata* presumably occurred among neighboring plants. Sphingids are strong flyers and are certainly capable of travel between *N. attenuata* populations, which are not widely separated. In 2001, there were three small roadside populations and one large population (DI) within about 30 km of the Hwy 91 burn. The results of the array experiments, however, suggest that the moths accumulate little pollen on their mouthparts. The chances that the moths will carry pollen between *N. attenuata* populations become very low considering that other plant species may be visited along the way.

The advantages to *N. attenuata* of attracting insect pollinators are unknown but could include the benefits associated with outcrossing. Whereas the likely adaptive value of self-compatibility is the assurance of reproduction when pollinator populations are unpredictable [31,32], outcrossing permits the production of variable offspring with adapted genotypes, which is considered particularly important for colonizing species in unpredictable environments [26,33,34]. Another putative benefit might be an increase in seed set and production through transfer of self-pollen by the activity of the insect visitor. Because some of the potential pollinators, the *Manduca* species, are also herbivores that can inflict substantial damage on the plants, the benefits must outweigh the

risks of advertisement to these visitors. Alternatively, though, the risks themselves may be quite low. In the case of *Manduca* species, two considerations lower the danger: there tend to be more male than female visitors to flowers in hawkmoth populations [13], and *Manduca* species usually lay their eggs on young (elongating) rather than fully flowering *N. attenuata* plants [4]. There is presumably no risk in attracting *H. lineata*, for the larvae do not feed on *Nicotiana* [7,35].

Advertisement for pollinators is in some respects not phenotypically flexible: the flowers are always whitish and tubular, and always open and produce odors in crepuscular hours. In contrast, *N. attenuata* exhibits a great deal of flexibility in its defenses against herbivory. Populations of herbivores, like those of pollinators, are unpredictable, and its sophisticated systems of induced chemical defenses reflect the costs of defense in terms of fitness and competitive ability [2]. More subtle changes in floral advertisement, however, suggest that the plant adjusts its responses in such a way as to manage the dilemma of advertisement to herbivores as well as pollinators. Though benzyl acetone emissions do not change, herbivory increases the amount of nicotine, a toxin and deterrent, in the corolla odor pool [12]. The production of flowers with exerted stigmas is altered by herbivory as well (Keâler, unpublished data; [19]). Our study did not provide a true outcrossing rate, and genetic demographic studies will have to be conducted in order to determine whether outcrossing has any significant evolutionary effects. Nonetheless, our demonstration that outcrossing can occasionally occur helps explain the maintenance of floral traits that attract insect visitors. Both the breeding and the defense system of *N. attenuata* represent adaptations to the unpredictable environment of an ephemeral species.

Methods

Source of transformed plants

Transformed *N. attenuata* plants homozygous for two frequently used reporter genes, hygromycin resistance and β -glucuronidase (GUS) expression, were created at the Max-Planck-Institut für Chemische Ökologie as described by Krügel *et al.* [36]. Briefly, the genotype that was transformed originated from seeds collected in Washington County, Utah, in 1988. Plants were transformed with *Agrobacterium tumefaciens* (LBA 4404) (Life Technologies-Gibco BRL, Eggenstein, Germany), with the pCAMBIA1301 plasmid (CAMBIA, Canberra, Australia) harboring the hygromycin antibiotic resistance gene (*hptII*) and *gus* from *E. coli*, both under control of a 35S cauliflower mosaic virus promoter. Transformants with single copy insertions were determined by segregation analysis of the T₁ generation and homozygous T₂ lines were identified by screening seedlings for hygromycin resistance and GUS expression.

Since both genes are inherited as dominant alleles, heterozygote seeds resulting from the cross of a homozygous pollen donor and a wild-type female are readily determined by germinating seeds on a sterile medium (Gamborg B5 in 0.6% Phytigel (Sigma-Aldrich) containing 20 mg/L hygromycin B (Duchefa, Haarlem)). The seeds were first sterilized in a solution of 0.1 g dichloroisocyanuric acid (Sigma-Aldrich) in 5 ml water with 50 μ l 0.5% Tween (Merck Eurolab) as wetting agent (5 min), and incubated for 1 h in a solution of 50 μ l 0.1 M GA₃ in EtOH (Duchefa, Haarlem) in 5 ml of liquid smoke (House of Herbs, Inc.; Passaic, New Jersey) diluted 1:50 in water to promote germination. The seeds were incubated for 5–7 d, until cotyledons appeared, in a climate chamber (16:8 l:d, 65% RH, 30°C). After another 10–14 d at 25°C, the surviving seedlings were also tested for GUS activity using a standard colorimetric assay [37]. The homozygous second-generation transformed plants, which were the sources of pollen used in the following experiments, were similarly identified by survivorship ratios on hygromycin media.

Field investigation of pollen transfer

Two large populations of *N. attenuata* were studied during the summer of 2001. Both were located in Washington County, Utah, one ("DI burn") near the intersection of Motoqua Road and the Beaver Dam Wash (37°18'38" N, 113°58'13"W, el. 1240 m) and the other ("Hwy 91 burn") along Highway 91 between St. George and the Arizona border (37°07'25"N, 113°49'47"W, el. 1500 m). Both sites had burned during the summer of 2000, according to Bureau of Land Management records (Cedar City, Utah). The studies were conducted between 20 June and 15 August 2001, when most plants in both populations were in flower.

Emasculated *N. attenuata* flowers in insect-free glasshouses do not set seed unless hand-pollinated [9]. To estimate the rate of pollen transfer in the field, an experiment was conducted at the Hwy 91 burn in which 206 flowers on 29 plants (mean 7.1 \pm 0.7 flowers/plant) in an approx. 1600 m² area were emasculated: any seed set that occurred could be attributed to pollen arriving from an external source. Flowers were selected that were about a day short of opening and were emasculated by making a slit from about halfway up the corolla through the rim, and removing the anther heads with a forceps [12]. Such flowers can be recognized by the tightly furled corolla, often not quite fully extended and yellowish rather than white at the tip; at this stage the anthers have not yet dehisced but are nearly mature. Any flowers in which the anthers had begun to release pollen, or in which the stigma was touched during the operation, were not used. The base and peduncle of the dissected flowers were marked with a dot of permanent ink and the plants were tagged with loops of white nylon yarn. Flowers and capsules in the

vicinity (within about 10 cm) were removed to minimize spurious pollination. Each flower was checked the evening after emasculation to be sure that it was otherwise intact. Upon opening, the emasculated flowers were nearly indistinguishable from untreated flowers in shape, size, and color, being only slightly wider at the cut rim. Moreover, emasculation does not alter flower volatile emissions [12]. It was therefore assumed that attractiveness to pollinators was retained.

To determine if emasculation influenced seed set by eliminating the self-pollen supply, an additional 37 flowers on the 29 plants were emasculated and pollinated by hand. Pollen (approximately one anther-equivalent) was transferred from the anthers of nearby plants to the stigmas of the dissected flowers in the tip of a Pasteur pipette [12]. To eliminate wind pollination and apomixis as additional mechanisms of seed set in the emasculated plants (cf. [38]), a pollinator-exclusion control was also established. Flowers were emasculated on an additional 5 plants: 34 received no pollen and 23 were hand pollinated. The plants were then enclosed in polyester Fibe-Air Plant Sleeves (KleenTest, Milwaukee, Wisconsin). The sleeves were removed after one week, i.e., after seed set would have occurred and the flowers were no longer receptive to pollinators.

The seed capsules were collected promptly upon maturation, 17–21 d after flowering. At the same time, 20 capsules were collected from 20 untreated plants in order to compare seed number and viability with the emasculated flowers. All seeds from collected seed capsules were screened for viability by sprouting them on sterile media as described above.

Two parallel experiments were conducted in the field to determine the utility of genetic markers in monitoring pollen transfer and to measure the competitive ability of pollen from transformed plants. In the first, pollen from transformed plants was mixed with pollen from wild plants at the DI burn; in the second, the transformed pollen was mixed with pollen from plants at the Hwy 91 burn. Transformed plants from homozygous lines were grown in a shade-house enclosure at the Lytle Preserve, Santa Clara, Utah. Leaf discs taken from the flowering plants were assayed for GUS activity to confirm that the plants were transformed. Anthers were collected from flowers that appeared to be one day short of opening. Between 50 and 100 anthers were mixed in glass vials at the following ratios: 100% anthers from field grown plants, 1:9 transformed: field, 1:1, 9:1, and 100% transformed. The vials were placed, open, in a sealed plastic container containing silica-gel dessicant. Most of the anthers dehisced within a few hours. After 2 days, the pollen in each vial was mixed thoroughly with a metal spat-

ula and applied to emasculated flowers at the Hwy 91 burn. The pollen mixture using wild pollen from the DI burn was applied to 25 plants, representing 25 replicates of the set of ratios, while the pollen mixture from the Hwy 91 burn was applied to 20 plants.

In 2001, unusually large numbers of white-lined sphinx moths (*H. lineata*) were present in *N. attenuata* habitats. Hundreds were seen visiting flowers on most evenings between mid-June and mid-August. Two measures were made of their activities. First, the rate at which they visited *N. attenuata* plants was estimated by counting the number of plants visited in 16 plots (5 × 5 m) during 10-min intervals. The counts took place during the peak evening flight period, which started 15 to 30 min before sunset and lasted until about 30 min after sunset. The plots varied in density, so a rough count was made of the number of plants in each. The number of moths visiting each plot and the number of plant visits were recorded. The 10-min period started when the first moth entered the plot. These observations were made between 1 July and 11 July at the Hwy 91 burn. Second, the movement patterns of moths were observed on the evenings of June 27 (DI burn, 20 moths), June 28 and June 29 (Hwy 91, 21 moths) as they moved through *N. attenuata* patches. The distances between plants (estimated by eye) and the numbers of flowers visited per plant were recorded. Moths were followed until they were lost from sight.

Laboratory investigation of pollen transfer by *Manduca sexta*

Genetic markers were used to determine whether adult *M. sexta* transfer pollen between *N. attenuata* plants. Captive moths visited arrays of wild-type and transformed plants; the appearance of the marker genes in the offspring of the wild-type plants would indicate that the visits resulted in outcrossing.

A preliminary study was performed to determine whether pollen from transformed plants could produce seeds when applied to intact flowers, i.e., which still had their full complements of self-pollen. A fine paintbrush was touched to the anthers of a flower on a transformed plant, then to the stigma of a flower on a wild-type plant. Forty wild-type flowers from 8 plants were treated.

Twenty-five *Manduca sexta* pupae (Carolina Biological Supply; Burlington, North Carolina) were kept at 9°C for three weeks after arrival and then transferred to a climate chamber (28°C, 80% RH, 16:8 l:d). Nineteen moths (12 ♀ and 7 ♂) emerged within 3d. Within 24 h of emergence (i.e., after the wings had hardened) each moth was transferred to a 2 × 2 × 2 m pollination arena constructed of white nylon mesh, in a glasshouse with conditions

approx. 25°C, 16:8 l:d. No nectar sources other than the experimental *N. attenuata* flowers were available.

Starting on the day on which the last of the 19 moths was transferred to the tent, a series of three experimental arrays was presented to the moths. The first array was designed to maximize movement between transformed and wild-type plants. It consisted of a 5 × 5 arrangement of alternating transformed (n = 13) and wild-type plants (n = 12), all approx. 75 cm tall. On the wild-type plants, older flowers and capsules that had already set seed were trimmed away, leaving 12–15 flowers open on each plant for the course of the experiment, and the transformed plants received similar cutting though some of the older flowers were left as pollen sources. The plants were 25 cm apart at the base, so that flowers on adjacent plants were close but not in contact. The moths were observed during the peak activity time that lasted for about 30 min around dusk. The identities of the plants visited were recorded during 15-min intervals. The moths appeared to fly normally, visiting series of 2–20 plants in sustained flights lasting several minutes. The females paused occasionally to lay eggs, which were removed from the plants every afternoon.

After four days, the first array was removed and replaced with a 3 × 3 array consisting of eight transformed plants surrounding one wild-type plant in the center. To avoid disturbing the moths, the rearranging took place during daylight hours, when the moths rested, inactive, on the sides of the cage. The plants were treated as above and were also 25 cm apart. It was predicted that if the moths accumulate pollen while visiting flowers, then there should be a greater proportion of transformed seedlings among the wild-type plant's offspring, given the greater ratio of transformed to wild-type flowers compared to the first array. The moths' activities were also checked while the second array was in place, to be sure that they were still visiting the flowers. After three days, this array was removed and replaced with a 4 × 4 array of alternating transformed and wild-type plants. The moths were removed from the tent before these plants went in. The third array provided a control for pollen transfer not mediated by the insects: if no transformed seeds were found in the wild-type plants, further support would be provided for the hypothesis that the moths were responsible for pollen transfer in the first two arrays, whereas a positive result would suggest that pollen can be transferred by gravity or air movement. After removal from the experimental arrays, the flowers that had been open during the experiments were marked with a streak of permanent ink on the petiole. The seeds matured in 15–17 days and were promptly screened for hygromycin resistance and GUS expression.

Authors' contributions

Both authors participated in the design and coordination of the study. KS carried out the laboratory analyses and glasshouse and field experiments, and drafted the manuscript. Both authors participated in revising the manuscript and read and approved the final version.

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