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ASYMMETRIC EFFECTS OF LOSS AND GAIN OF A FLORAL TRAIT ON POLLINATOR PREFERENCE

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Shifts in pollination syndromes involve coordinated changes in multiple floral traits. This raises the question of how plants can cope with rapid changes in pollinator availability by the slow process of accumulation of mutations in multiple genes. Here we study the transition from bee to hawkmoth pollination in the genus *Petunia*. Interspecific crosses followed by single locus introgressions were used to recreate putative intermediate evolutionary stages in the evolution of moth pollination. The effect of the loss/gain of petal color was asymmetric: it had no influence on the established pollinator but enhanced visitation by the new pollinator. Therefore, shifts in pollination syndromes may proceed through intermediate stages of reduced specialization and consequently enhanced reproductive assurance. The loss of petal color in moth-pollinated *Petunia* involves null mutations in a single regulatory gene, *An2*. Such simple genetic changes may be sufficiently rapid and frequent to ensure survival during pollinator failure.

KEY WORDS: **Flower color, molecular evolution, near isogenic lines, plant–insect interactions, pollinator preference, transcription factor AN2.**

Pollinators recognize floral characters such as petal color, flower morphology, scent production as well as nectar volume, concentration, and composition (Fenster et al. 2004; Kay and Sargent 2009). These suites of floral traits, called "pollination syndromes" have been observed in unrelated plant taxa, suggesting independent evolutionary scenarios and strong selective pressures. Failure of an established pollinator due to disease or invasion of a more attractive competitor plant species may happen quite suddenly (Roels and Kelly 2011). This problem has received increased attention in connection with the world-wide decline in bee populations and as part of the debate on climate change. Plants can adapt to the loss of pollinator by becoming self-fertilizing, by becoming perennial or by attracting a new pollinator (Kalisz and Vogler 2003; Charlesworth 2006; Fenster and Marten-Rodriguez 2007). Shifts in pollination syndromes have happened frequently in many different angiosperm families, for instance in the Solanaceae there is evidence for at least 10 independent shifts to hummingbird pollination (Knapp 2010). How can a plant genetically adapt to a new pollinator and how can this adaptation be sufficiently rapid to survive? Differences between pollination syndromes involve multiple traits, each of which is specified by multiple genes. It must take a considerable number of generations to acquire multiple individual mutations and accumulate them in a single plant, too slow to cope with the sudden loss of a pollinator.

In most cases, it remains unclear how individual floral features or combinations of these features are integrated by the animal's sensory system and how they affect pollinator behavior. With such complex combinatorial possibilities, the difficulty lies in assessing precisely the role of single floral features. The role of petal color has been intensely studied from all different angles. Experienced bees prefer blue and yellow colors, and violet was the most easily learnt color (Gumbert 2000). Similarly, the diurnal hawkmoth *Macroglossum stellatarum* has an innate preference for blue, but can be trained to favor yellow color (Balkenius and Kelber 2006). The nocturnal hawkmoth *Manduca sexta* prefers white flowers despite an innate preference for blue (Goyret et al. 2008). Therefore, color preference can be changed by learning. It

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should also be noted that most hawkmoth-pollinated flowers are white to the human eye, but because most of them absorb UV light the reflected light will differentially activate the three photoreceptors of these insects (White et al. 1994; Kevan et al. 2001). As color perception decreases the eye's absolute sensitivity, there must be strong ecological constraints to conserve trichromatic vision with nocturnal habits (Kelber et al. 2002). Thus, flower color is an important and complex cue for pollinators.

One of the recent themes in pollination biology has been to genetically disentangle pollination syndromes into their component traits and to study them individually in defined genetic backgrounds. Genetic and ecological studies may then answer how pollinator shifts are conditioned by natural allelic variation in floral traits. A classic example is the YUP locus, which controls the deposition of yellow carotenoid pigments in the petals of *Mimulus*. Reciprocal introgressions of YUP into *Mimulus lewisii* (pollinated by bumblebees) and *Mimulus cardinalis* (pollinated by hummingbirds) triggered a significant shift of pollinator preferences in both directions (Bradshaw and Schemske 2003). The YUP locus has not yet been identified at the molecular level and it is not known whether it encodes a single gene. If the YUP locus is more complex, a change of phenotype would presumably entail the accumulation of multiple mutations.

The role of floral color genes has been widely studied in several species, and changes in the expression and function of structural as well regulatory genes associated with pollinator visitation have been documented (Quattrocchio et al. 1999; Streisfeld and Rausher 2009a,b; Des Marais and Rausher 2010; Hopkins and Rausher 2011). The genus *Petunia* contains species with pollination syndromes adapted to bees, hawkmoths, and hummingbirds. Bee pollination in the *Petunia integrifolia* group is ancestral, whereas moth-pollinated *Petunia axillaris* is a derived species (Ando et al. 2005; Kulcheski et al. 2006; Stehmann et al. 2009). When the two species grow in sympatry, no hybrids are formed largely because of different pollinator preference (Dell'Olivo et al. 2011). The genetics, biochemistry, and molecular biology of flower color in *Petunia* have been studied in great detail, making it one of the best known pathways of secondary metabolism in plants (Tornielli et al. 2009). Interspecific crosses identified three loci responsible for the differences in petal color between the purple *P. integrifolia* and the white *P. axillaris* (Wijsman and Vandenberg 1982; Wijsman 1983; Quattrocchio et al. 1999). These were mapped to known mutations in *Petunia hybrida*. *Hf1* encodes a biosynthetic enzyme that modifies color (Shimada et al. 2001; Chen et al. 2007), whereas *AN2* encodes an MYB transcription factor (Quattrocchio et al. 1999) responsible for the late steps in anthocyanin biosynthesis. Flavonol synthase (FLS) is the key enzyme in the biosynthetic pathway that leads to the production of UV-absorbing flavonols. Flavonols and anthocyanins share dihydroflavonols as common precursors and therefore FLS expression may affect visible color through metabolic competition (Huits et al. 1994).

Quattrocchio et al. (1999) found evidence for at least three independent nonsense or frameshift mutations in the *P. axillaris AN2* coding sequence, suggesting that the loss of petal color happened several times. We followed up on this work by analyzing the expression of AN2 in 35 wild accessions (Hoballah et al. 2007). In all natural accessions, the loss of purple color was associated with inactivating mutations in the *AN2* coding region, confirming the previous findings (Quattrocchio et al. 1999) and showing that these mutations were not due to propagation in culture but were present in nature. Furthermore, we asked what influence the modification of AN2 might have on pollinator behavior. To this end we complemented the defective *AN2* gene in *P. axillaris* by introducing a functional *AN2* allele. The transgenic plants had anthocyanin-containing flowers but were otherwise indistinguishable from the nontransgenic parent. Choice assays under controlled conditions showed that hawkmoths preferred the white parent whereas bees were significantly more attracted to the colored transgenic plant. Thus, a single gene can have a significant effect on pollinator behavior (Hoballah et al. 2007). These experiments had a number of limitations. First, the experiments were performed only in the background of *P. axillaris* and not in the background of the ancestral bee-pollinated species. Second, the experiments were performed in an atypical accession of the *P. axillaris parodii* subspecies which does not produce flavonols and consequently does not absorb UV light. Third, transgenic plants cannot be released in nature precluding experiments in natural habitats. Here, we crossed purple UV-reflecting bee-pollinated *P. inflata* with a hawkmoth-pollinated, white, UV-absorbing *P. axillaris* and used the F1 plants to construct near isogenic lines that differed from the parents at the *AN2* locus. These plants were used in behavioral experiments in the greenhouse and at a sympatric location in the native habitat. We show that the acquisition of a single locus has more effect on visitation by a new pollinator than its loss has on the established pollinator. This suggests that the first step toward attracting a new pollinator can be made without compromising visitation by the established pollinator.

Materials and Methods

PLANT MATERIAL

Petunia axillaris axillaris N and *P. integrifolia inflata* S6 were kindly provided by Ronald Koes (Vrije Universiteit Amsterdam). They are referred to as *P. axillaris* and *P. inflata*, respectively. The plants were maintained in a greenhouse under 16 h : 8 h light–dark and a minimum temperature of approximately 20°C. Plants were grown in commercial soil (70% Klasman substrate, 15% Seramis clay granules, 15% quartz sand) and were

fertilized once a week. For volatile and nectar measurements, the plants were maintained in a growth room with an altered light–dark cycle (light 00 h–15 h, 22°C; dark 15 h–00 h, 17°C. Near isogenic lines (NILs) segregating for the An2 locus were obtained by combinations of backcrosses (BC_n) and selfings (F_n). The An2 genotype was determined using 25 allele-specific CAPS markers (http://www.botany.unibe.ch/deve/caps/index.html). The pedigree of *P. axillaris*$^{An2\ P.\ inflata\ S6}$ is an $F_1BC_5F_3$. The line is referred to as An2$^+$ *P. axillaris*. Except for An2 and the two linked markers C4H1b and 3-KAT, all markers were homozygous for the recurrent parent *P. axillaris*.

P. inflata$^{An2\ P.\ axillaris\ N}$ was an $F_1BC_1F_1BC_4F_3$. The line is referred to as An2$^-$ *P. inflata*. All markers were homozygous for the recurrent parent *P. inflata*, except for AN2 and C4H1b and 3-KAT.

INSECTS

Manduca sexta hawkmoths (strain Yamamoto) were obtained as female pupae from the North Carolina State University insectary or from Philipps-Universität (Marburg, Germany). They were put in a bugdorm tent (Bugdorm 2; MegaView Science Co., Ltd., Taichung, Taiwan) until emergence (light 2 h–17 h, dark 17 h–2 h, 25°C, 65% relative humidity). They were used for experiments from 1 to 7 days after emergence and were only fed with water. Bumblebees were obtained from Andermatt Biocontrol (Grossdietwil, Switzerland). The hives are made of a plastic container protected by a cardboard box, with a sugar solution tank at the bottom. They were maintained in the greenhouse, inside a bugdorm tent.

Behavioral assays

The behavioral assays under controlled conditions were carried out with hawkmoths inside a cage (*H* 144 cm × *W* 248 cm × *L* 368 cm) made of insect net (Migros M-Garten) sustained by an aluminum structure. This cage was built inside a dedicated petunia greenhouse of the Botanical Garden of the University of Bern, and so was saturated by *Petunia* fragrance at night, which was a good elicitor for hawkmoths flight (Raguso and Willis 2002).

The plants were put in an alternate order. The position of the plants was changed each day. Eight plants were used; four for each genotype, and each of the lines had the same number of flowers. The entrance site of the moths varied between each assay, to minimize the impact of the first flower seen on the first choice. The observations took place at dusk, starting time varying between 17 h (in winter) and 21 h (in summer). End time varied depending on the hawkmoths: when none were flying anymore in the separated cage containing naïve moths, the experiment stopped. Moths were taken one by one and released in the experimental cage for 5 min. They could fly freely during this time and their behavior bouts (feeding and duration of feeding events) were recorded. At the end of the experiment, they were put in another cage for experienced moths.

In the case of the *P. inflata* background (An2$^-$ *P. inflata* and *P. inflata*) behavioral assays, hawkmoth attraction in this "bee background" might be poor. Thus, the experiment was modified as follows: after the end of each assay with the *P. inflata* background plants, plants from the two lines in *P. axillaris* N background were added and pollinator behavior was observed for another 5 min.

The bumblebee behavioral assays were carried out in the same cage, but during the day. Each hive was used two to three times. Observations began between 10 and 11 a.m., depending on the sunlight, and lasted for 2 h. They were carried out with a minimum temperature of 20°C inside the greenhouse. Observations were also replicated during the afternoon, beginning between 13 and 14 h and finishing around 16 h 30 min. One hive was put in the cage at least 30 min before the beginning of the experiment, to let the bees adjust to the experimental area. At the beginning of the experiment, the bumblebees were allowed to leave and enter the hive freely during the observations. The plants were displayed in an alternate pattern and the two lines had the same number of flowers. We recorded the number of pollination events (the insect clearly touching the anthers with the head) separately for each plant. The An2$^+$ *P. axillaris* was compared to *P. axillaris* and An2$^-$ *P. inflata* to *P. inflata* following exactly the same experimental protocol.

Wild pollinator observations were carried out in Las Canas, a sympatric site located close to Fray Bentos, Rio Negro, Uruguay (33°9′59.86″S 58°21′25.10″W) in January 2009. Both *P. axillaris* and *P. integrifolia* plant populations were found growing around and inside a holiday village. The plants were arranged in an alternate pattern and the two compared lines had exactly the same number of flowers. The observations took place from 9 h until 12 h. The time, the identity of the pollinator, and its behavior were recorded for each visit. Feeding events were recorded. Nocturnal visitation was assessed from dusk to midnight but only few moths were observed, presumably due to drought conditions.

STATISTICS

Data were first analyzed to check the parametric test assumptions, normality, and homogeneity of variances (homoscedasticity). The normality of the distribution was determined by a Shapiro–Wilt test and the homoscedasticity with Levenne's test. If both assumptions were respected, a *t*-test (for the comparison of two samples) or an ANOVA was used. The latter was followed by a post hoc test (Students–Newman–Keuls) to distinguish between significantly different groups. When the requirements of the parametric tests were not met, nonparametric tests were carried out: the Friedman test for more than two groups of samples, and for two groups of

samples, the Wilcoxon signed rank test. First choice behavioral assays were tested using χ^2-test.

PHENOTYPIC MEASUREMENTS

Visible and UV color. Flavonols and anthocyanins were extracted overnight from petal tissue disks of 8 mm diameter in 1 mL of water:methanol:acetic acid mix of 7:2:1. Absorption measurements represent integrated spectrophotometer values of wavelengths from 315 to 378 nm for flavonols and from 445 to 595 nm for anthocyanins. For UV-absorption measurement, we also used a Digital Single Lens Reflex standard objective and a UV pass filter. The flowers were deposited on a black support and pictures were taken outside.

Floral tube length and corolla projected area differ significantly between *P. axillaris* and *P. inflata* (Galliot et al. 2006). For floral tube length we measured D1, the section of the tube in which petals and anther filaments are fused following Stuurman et al. (2004). For the determination of the projected area we followed the method described in Venail et al. (2010).

Nectar quantity was measured by the following method: on the first day, all flowers were removed from the plant. On the second day, the flowers that opened during the interval were marked. On the third day, flowers were cut 1.5 cm above the contact between petals and sepals, and the cut base of the tube put in a 500 μL eppendorf tube with a hole in the bottom (the ovary being placed up). All this was put in a second 1,500 μL eppendorf tube and spun 30 sec in a microfuge. The quantity of nectar was measured by weighing.

Scent. Volatiles were collected by placing plants in odorless Nalophan cooking bags (Kalle GmbH). The volatiles were retained on SuperQ filters and analyzed with a gas chromatograph (Agilent 7890A) coupled to a mass spectrometer detector (Agilent 5975C) as described (Hoballah et al. 2005; Degen et al. 2012). For details see http://www.botany.unibe.ch/deve/research/projects/pollinator.php.

Results

To study the effect of substitution of the *AN2* gene in each background, reciprocal introgressions were performed; *P. axillaris* plants with an introgressed $An2^{P.\ inflata}$ locus and *P. inflata* plants with an introgressed $An2^{P.\ axillaris}$ locus were produced. These near isogenic lines (NILs) are further referred to as "$An2^+$ *P. axillaris*" and "$An2^-$ *P. inflata*" (Fig. 1). The $An2^+$ *P. axillaris* flowers were lighter purple than those of *P. inflata* indicating that additional loci are required to obtain full color (Fig. 1A). Similarly, the $An2^-$ *P. inflata* flowers had a white corolla limb but the tube retained anthocyanins. This was expected as tube color is under the control of the AN4 locus on chromosome VII (Wijsman and Vandenberg 1982). Scanning EM analysis indicated that all four accessions had conical cells in their epidermis, making it unlikely that mechanical differences play a role (data not shown). In all other traits of potential relevance to pollinators differences between the NILs and their recurrent parents were either insignificant or minor and unlikely to influence pollinator behavior. The exception is corolla projected area, which was 28% smaller in the $AN2^-$ *P. inflata* NIL (Fig. 1E). In previous experiments with *P. axillaris* plants that differed in corolla width by 57%, we found that hawkmoths have a preference for flowers with a larger projected area (Venail et al. 2010) and therefore in the experiments presented here the moths might have a small bias against the NILs. Note that this effect would counteract a positive effect of the loss of AN2.

RESPONSE OF HAWKMOTHS TO PLANTS DIFFERING IN AN2 FUNCTION

When $An2^+$ *P. axillaris* and the isogenic parent *P. axillaris* were presented to the hawkmoth *M. sexta*, the insects showed no significant preference for the number of feeding events (Fig. 2A), nor for the first choice (15 moths chose $An2^+$ *P. axillaris* and 14 moths chose *P. axillaris*, χ^2-test, $P = 0.85268$). The mean time spent feeding per flower was also similar (Fig. 2C).

When we assessed An2 in the *P. inflata* background, the moths preferentially chose the $An2^-$ *P. inflata* flowers, both expressed as the mean number of feeding events (Fig. 2B) and as first choice (15 vs. 3 during the initial 5 min; χ^2-test, $P = 0.005$). There was no significant difference between times spent feeding per visit (Fig. 2D).

Although loss of AN2 function would shift the color of the flower closer to the classical hawkmoth pollination syndrome, other traits such as scent and morphology do not conform. This raises the question to what extent plants in the *P. inflata* background could compete with *P. axillaris* plants, which display the "classical" hawkmoth pollination syndrome. Therefore, we subsequently added the two isogenic *P. axillaris* lines to the experiment (Fig. 2E). In this second experimental phase, the moths clearly showed their preference for the *P. axillaris* versus the *P. inflata* background. Note that the moths spent ten times more time feeding on *P. axillaris* background plants than on the *P. inflata* background plants (Fig. 2F). Thus, although the $An2^-$ *P. inflata* plants were preferred over the *P. inflata* parent, they were less attractive than either *P. axillaris* genotype. Therefore, we conclude that the modification of a single trait can change pollinator preference but that the ensemble of traits provides a clear relative advantage.

RESPONSE OF BEES TO PLANTS DIFFERING IN AN2 FUNCTION

We next tested the response of the two pairs of isogenic lines to bee pollinators. The natural pollinators of *P. inflata* and related species

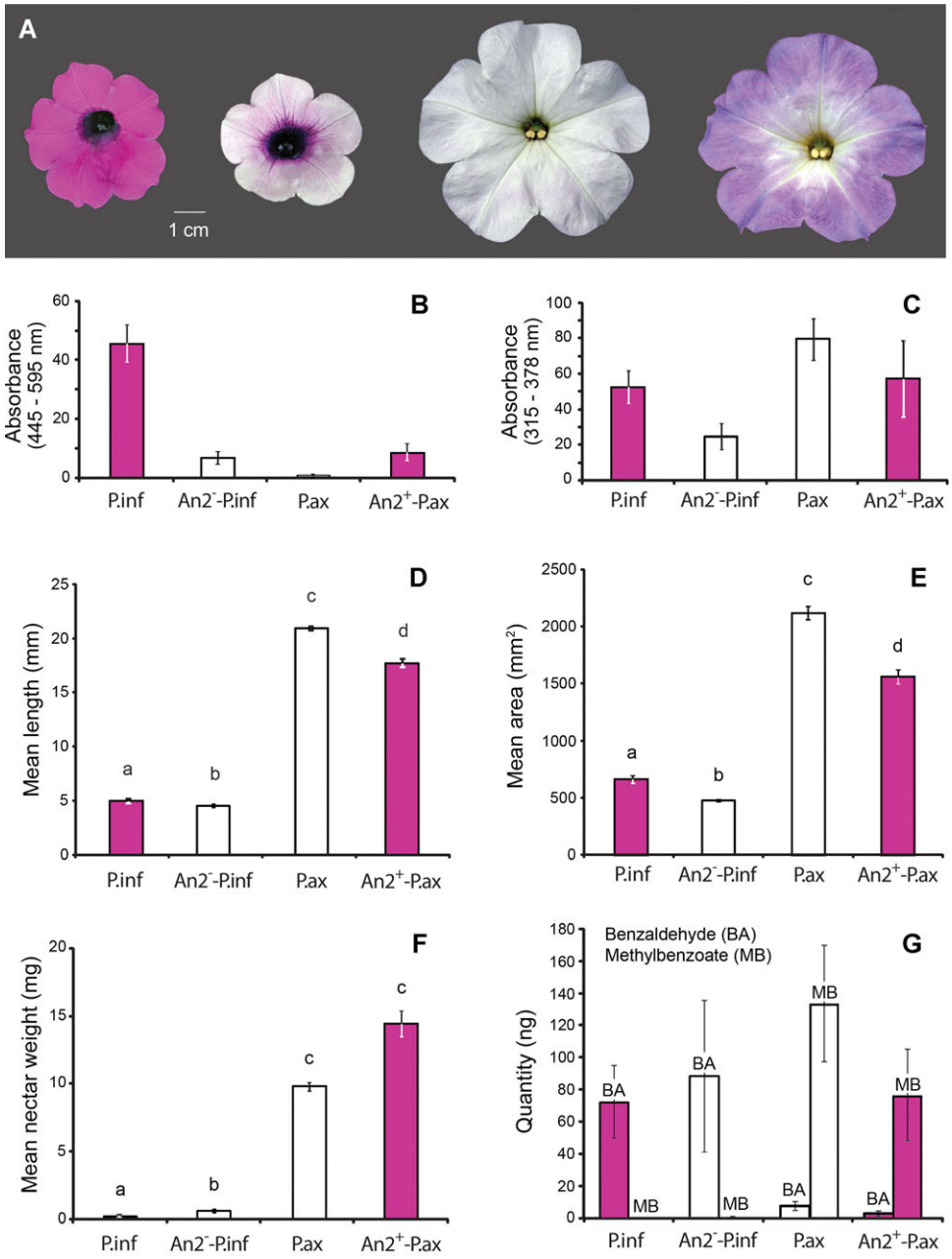

Figure 1. Reciprocal introgressions of the AN2 locus differ in petal color. (A) From left to right: *Petunia inflata* parental line; near-isogenic An2^{-} *P. inflata*; *Petunia axillaris* parental line; near-isogenic An2^{+} *P. axillaris*. Scale bar = 1 cm. (B) Visible color. Absorption measurements represent integrated spectrophotometer values of wavelengths from 445 to 595 nm. (C) Ultraviolet color. Absorption measurements represent integrated spectrophotometer values of wavelengths from 315 to 378 nm. (D) Floral tube length. The D1 section of the tube, in which petals and anther filaments are fused, was measured. (E) Projected corolla area (front view). An2^{+} *P. axillaris* flowers were smaller than *P. axillaris* (Mann–Whitney test: $Z = -5.109$; $P = 0.0001$). The An2^{-} *P. inflata* displayed a smaller petal area than *P. inflata* (Mann–Whitney test: $Z = -4.845$; $P = 0.001$). (F) Nectar quantity. (G) Scent. The two main volatiles identified by Hoballah et al. (2005), benzaldehyde and methyl benzoate, were measured by GC-MS.

are small solitary bees (Hoballah et al. 2007; Dell'Olivo et al. 2011). For practical reasons we performed the greenhouse experiments with the bumblebee *Bombus terrestris*, which are readily available. When *B. terrestris* were given the choice between An2^{+} *P. axillaris* and *P. axillaris*, they showed a significant preference for An2^{+} *P. axillaris* (Fig. 3A). The bumblebees mainly collected pollen, their tongue being too short to reach the nectar at the bottom of the long and thin tubes. When tested for the effect of AN2 function in the *P. inflata* background, the bumblebees showed no significant preference for An2^{-} *P. inflata* compared to *P. inflata*,

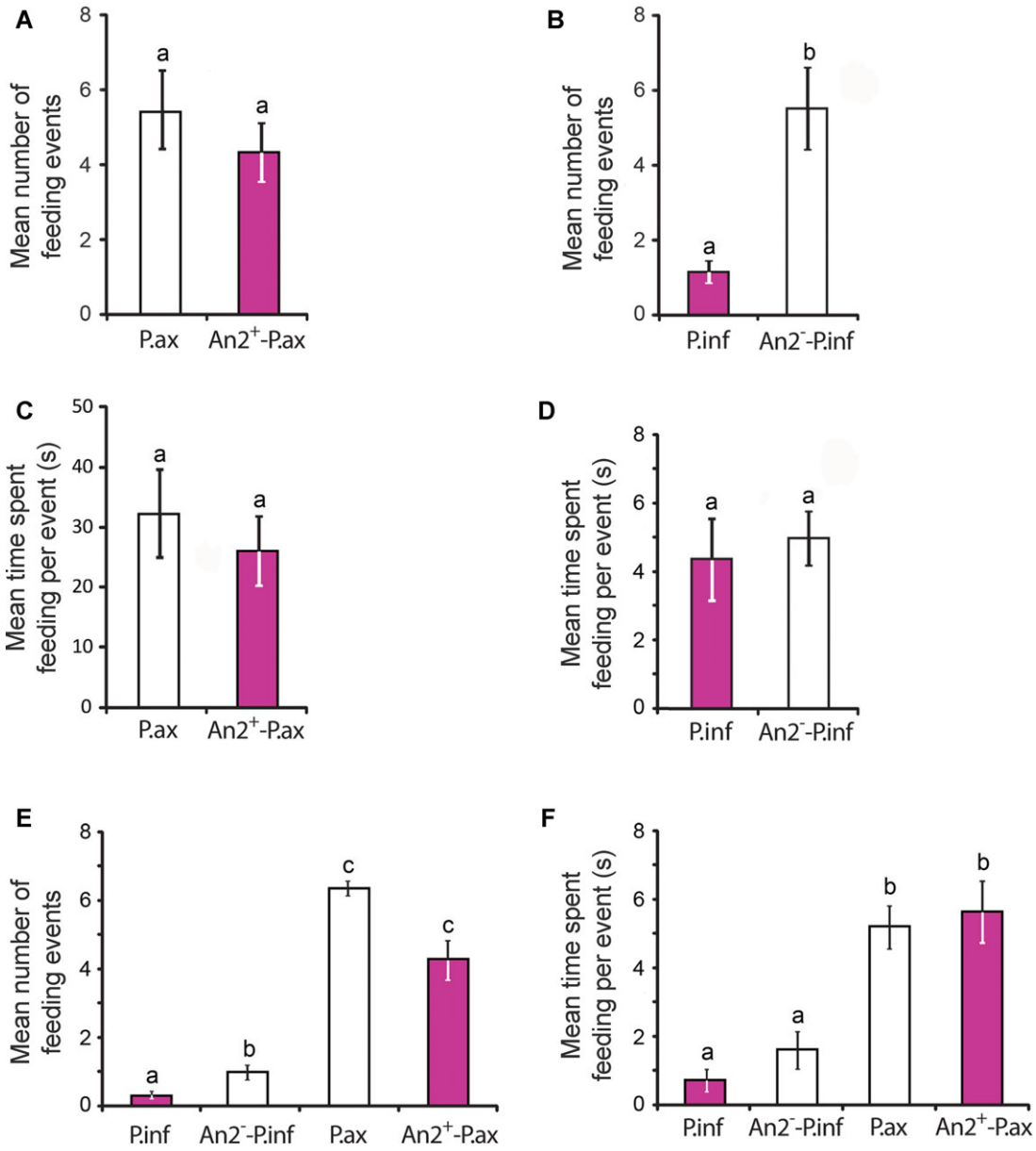

Figure 2. **Presence/absence of functional AN2 affects hawkmoth preference in the *Petunia inflata* but not in the *Petunia axillaris* genetic background. (A) Choice assay. Naive *Manduca sexta* were exposed to *P. axillaris* and An2$^+$ *P. axillaris* during 5 min. Wilcoxon signed rank test, $Z = -0.893$, $P = 0.372$, $N = 29$. (B) Choice assay. Naive *M. sexta* were exposed to *P. inflata* and An2$^-$ *P. inflata* during 5 min. Wilcoxon signed rank test, $Z = -3.275$, $P = 0.001$, $N = 18$. (C) Mean time spent feeding per hawkmoth and per event during choice assay depicted in panel (A). Wilcoxon signed rank test, $Z = -0.238$, $P = 0.812$, $N = 29$. (D) Mean time spent feeding per hawkmoth and per event during choice assay depicted in panel (B). Wilcoxon signed rank test, $Z = -0.724$, $P = 0.469$, $N = 18$. (E) Choice assay. *Manduca sexta* were exposed to *P. inflata*, An2$^-$ *P. inflata*, *P. axillaris*, and An2$^+$ *P. axillaris*. After the choice assay depicted in panel (B), the two *P. axillaris* lines were added to the experiment and during a second period of 5 min, the moths could choose between the four genotypes. Friedman test, $\chi^2 = 52.179$, $P = 0.001$, $N = 26$. (F) Mean time spent feeding per hawkmoth and per event during the choice assay depicted in panel (E). Friedman test, $\chi^2 = 36.528$, $P = 0.001$, $N = 26$.**

for pollination events (Fig. 3B). Bumblebees collected both pollen and nectar, in contrast to the assays with the *P. axillaris* NILs.

For field experiments we selected a site where both plant species can be found. Such sympatric sites are fairly rare and the abundance, and even the presence, of both species may vary considerably from year to year (Dell'Olivo et al. 2011). Moreover, the presence of pollinators, especially hawkmoths, may also be highly variable. In fact, over the past few years hawkmoths have been rare in our study sites in Uruguay. Therefore our field observations have been restricted to visits by bees. As in the greenhouse

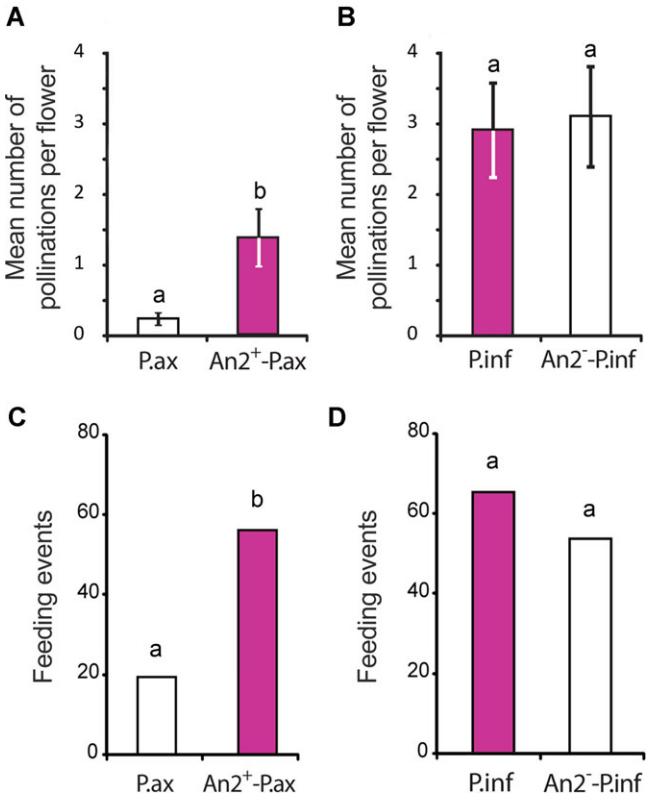

Figure 3. Presence/absence of functional AN2 affects bee preference in the *Petunia axillaris* but not in the *Petunia inflata* genetic background. (A) Choice assay. Naive *Bombus terrestris* were exposed to *P. axillaris* and An2$^+$ *P. axillaris*: mean number of pollination events per flower. Wilcoxon signed rank test, $Z = -4.816$, $P = 0.001$, $N = 4$ hives. (B) Choice assay. Naive *Bombus terrestris* were exposed to *P. inflata* and An2$^-$ *P. inflata*: mean number of pollination events per flower. Wilcoxon signed rank test, $Z = -0.635$, $P = 0.525$, $N = 4$ hives. (C) Behavioral response of wild solitary bees to *P. axillaris* and An2$^+$ *P. axillaris*: absolute number of feeding events. χ^2-test: 17.053, $P = 0.001$, $N = 7$ days of observation. (D) Behavioral response of wild solitary bees to *P. inflata* and An2$^-$ *P. inflata*: absolute number of feeding events. χ^2-test: 1.180, $P = 0.277$, $N = 7$ days of observation.

experiments, bees had a strong preference for AN2$^+$ *P. axillaris* plants compared to the *P. axillaris* parent, whereas they visited the two *P. inflata* genotypes at equal frequencies (Fig. 3C, D). Thus, we conclude that gain of AN2 function in the *P. axillaris* background changes the preference of the bees but the loss of AN2 function in the *P. inflata* background does not.

Discussion

Shifts between pollination syndromes have occurred frequently and are likely to be adaptations to changes in pollinator availability. Such shifts are genetically complex involving multiple genes underlying distinct floral traits. This raises a question of time scales. Despite the accumulating evidence for the involvement of genes of major effect (Bradshaw and Schemske 2003; Hoballah et al. 2007; Klahre et al. 2011), it is hard to imagine how a complete shift to a new pollination syndrome could be rapid enough to cope with the sudden loss of an established pollinator. Here we show that presumptive intermediate stages in the shift from bee to moth pollination have the potential to reduce specialization and thereby enhance reproductive assurance.

The *Petunia* lines used in this study are reciprocal introgressions of the AN2 locus on chromosome VI into the bee-pollinated *P. inflata* and the hawkmoth-pollinated *P. axillaris* as the recurrent parents. In this set of four genotypes, the two evolutionary endpoints are defined by the two parents, the ancestral *P. inflata* and the derived *P. axillaris*, whereas the introgression lines represent potential intermediate stages in the shift from a bee to a moth pollination syndrome. The preferences of the bee and hawkmoth pollinators for these four genotypes (Figs. 2, 3) can be summarized as follows. Bees preferred the colored An2$^+$ *P. axillaris* introgression line over the white isogenic parent. However, they showed no preference when choosing between the two *P. inflata* lines. This was observed with European bumblebees in the greenhouse as well as with natural pollinators, solitary bees, in a sympatric site in Uruguay. Conversely, the hawkmoth *M. sexta*, a natural pollinator of *P. axillaris*, preferentially chose the An2$^-$ introgression over the purple *P. inflata* parent but did not discriminate between *P. axillaris* and its colored AN2$^+$ sibling. Thus, the effect of An2 genotype on pollinators was asymmetric in both backgrounds: loss of color in *P. inflata* had little influence on bees but attracted a new pollinator, whereas the gain of color in *P. axillaris* had little effect on its established moth pollinator but enhanced visitation by bees. Because feeding time was similar between NILs and their recurrent parents we assume that the loss/gain of color had no effect on the efficiency of pollen transfer and that therefore enhanced visitation is likely to correlate with enhanced seed set.

We hypothesize that in AN2$^-$ *P. inflata* the bees continue to rely on redundant cues such as morphology, whereas for the hawkmoth the presence of a single favorable cue piques their attention. Moths continue visiting the An2$^+$ *P. axillaris* plants because of attractive scent, morphology, and UV absorbance cues, whereas for the bees the single favorable cue visible color makes a difference. Thus, modification of a single trait changes pollinator preference only in the absence of other favorable traits. Such redundant favorable cues serve as a buffer against the loss of a single cue. This hypothesis is in line with our previous observations in which we compared a transgenic *P. axillaris parodii* S7::AN2$^+$ line with the white isogenic parent (Hoballah et al. 2007). The inbred laboratory accession *P. axillaris parodii* S7 differs from *P. axillaris* N in that it does not produce UV-absorbing flavonols (Guebitz et al. 2009). As lack of UV absorbance reduces flower visibility to nocturnal moths (White et al. 1994;

Kevan et al. 2001; Kelber et al. 2002). *Petunia axillaris parodii* S7 is likely to be less attractive to hawkmoths than *P. axillaris* N. In this "compromised" genetic background, the moths displayed a clear preference for the white genotype versus the colored transgenic. Thus, the loss of two favorable cues (no flavonols/presence of anthocyanins) reduced visitation by the established pollinator. High specialization will enhance the efficiency of pollen transfer but also reduce the number of flower visits and thereby increase pollen limitation. This implies that specialized flowers are more vulnerable to fluctuations in pollinator availability (Barrett 1996; Fenster et al. 2004). Our data suggest that shifts in pollination syndromes can proceed through intermediate stages with relaxed pollinator specificity thereby enhancing reproductive assurance. The multiple independent losses of AN2 function documented in natural populations suggest that loss of anthocyanins may have been rapid and common.

AN2 is an MYB-type transcription factor that regulates the late steps in the anthocyanin biosynthetic pathway. Because the shift from purple to white flower corolla, a key step in the transition from bee to moth pollination, involves the inactivation of AN2, this gene can be designated a speciation gene. Analysis of genetically defined inbred lines that differ in AN2 function allowed us to study the effect of this locus on pollinator behavior. The application of next generation sequencing brings the identification and functional analysis of additional speciation genes within reach in *Petunia* and many other species.

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LITERATURE CITED

Ando, T., H. Kokubun, H. Watanabe, N. Tanaka, T. Yukawa, G. Hashimoto, E. Marchesi, E. Suarez, and I. L. Basualdo. 2005. Phylogenetic analysis of Petunia sensu Jussieu (Solanaceae) using chloroplast DNA RFLP. Ann. Botany 96:289–297.

Balkenius, A., and A. Kelber. 2006. Color preferences influences odor learning in the hawkmoth, *Macroglossum stellatarum*. Naturwissenschaften 93:255–258.

Barrett, S. C. H. 1996. The reproductive biology and genetics of island plants. Philos. Trans. R. Soc. Lond. Ser. B-Biol. Sci. 351:725–733.

Bradshaw, H. D., and D. W. Schemske. 2003. Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. Nature 426:176–178.

Charlesworth, D. 2006. Evolution of plant breeding systems. Curr. Biol. 16:R726–R735.

Chen, S., K. Matsubara, T. Omori, H. Kokubun, H. Kodama, H. Watanabe, G. Hashimoto, E. Marchesi, L. Bullrich, and T. Ando. 2007. Phylogenetic analysis of the genus Petunia (Solanaceae) based on the sequence of the Hf1 gene. J. Plant Res. 120:385–397.

Degen, T., N. Bakalovic, D. Bergvinson, and T. C. J. Turlings. 2012. Differential performance and parasitism of caterpillars on maize inbred lines with distinctly different herbivore-induced volatile emissions. PLoS ONE 7:e47589–e47589.

Dell'Olivo, A., M. E. Hoballah, T. Guebitz, and C. Kuhlemeier. 2011. Isolation barriers between Petunia axillaris and Petunia integrifolia (Solananceae). Evolution 65:1979–1991.

Des Marais, D. L., and M. D. Rausher. 2010. Parallel evolution at multiple levels in the origin of hummingbird pollinated flowers in Ipomoea. Evolution 64:2044–2054.

Fenster, C. B., and S. Marten-Rodriguez. 2007. Reproductive assurance and the evolution of pollination specialization. Intl. J. Plant Sci. 168:215–228.

Fenster, C. B., W. S. Armbruster, P. Wilson, M. R. Dudash, and J. D. Thompson. 2004. Pollination syndromes and floral specialization. Annu. Rev. Ecol. Evol. Syst. 35:375–403.

Galliot, C., M. E. Hoballah, C. Kuhlemeier, and J. Stuurman. 2006. Genetics of flower size and nectar volume in Petunia pollination syndromes. Planta 225:203–212.

Goyret, J., M. Pfaff, R. A. Raguso, and A. Kelber. 2008. Why do *Manduca sexta* feed from white flowers? Innate and learnt colour preferences in a hawkmoth. Naturwissenschaften 95:569–576.

Guebitz, T., M. E. Hoballah, A. Dell'Olivo, and C. Kuhlemeier. 2009. Petunia as a model system for the genetics and evolution of pollination syndromes. Pp. 29–49 *in* T. Gerats and J. Strommer, eds. Petunia: evolutionary, developmental and physiological genetics. 2nd ed. Springer, New York.

Gumbert, A. 2000. Color choices by bumble bees (*Bombus terrestris*): innate preferences and generalization after learning. Behav. Ecol. Sociobiol. 48:36–43.

Hoballah, M. E., J. Stuurman, T. C. J. Turlings, P. M. Guerin, S. Connetable, and C. Kuhlemeier. 2005. The composition and timing of flower odour emission by wild Petunia axillaris coincide with the antennal perception and nocturnal activity of the pollinator *Manduca sexta*. Planta 222:141–150.

Hoballah, M. E., T. Gubitz, J. Stuurman, L. Broger, M. Barone, T. Mandel, A. Dell'Olivo, M. Arnold, and C. Kuhlemeier. 2007. Single gene-mediated shift in pollinator attraction in Petunia. Plant Cell 19:779–790.

Hopkins, R., and M. D. Rausher. 2011. Identification of two genes causing reinforcement in the Texas wildflower *Phlox drummondii*. Nature 469:411–414.

Huits, H. S. M., A. G. M. Gerats, M. M. Kreike, J. N. M. Mol, and R. E. Koes. 1994. Genetic control of dihydroflavonol 4-reductase gene expression in *Petunia hybrida*. Plant J. 6:295–310.

Kalisz, S., and D. W. Vogler. 2003. Benefits of autonomous selfing under unpredictable pollinator environments. Ecology 84:2928–2942.

Kay, K. M., and R. D. Sargent. 2009. The role of animal pollination in plant speciation: integrating ecology, geography, and genetics. Ann. Rev. Ecol. Evol. Syst. 40:637–656.

Kelber, A., A. Balkenius, and E. J. Warrant. 2002. Scotopic colour vision in nocturnal hawkmoths. Nature 419:922–925.

Kevan, P. G., L. Chittka, and A. G. Dyer. 2001. Limits to the salience of ultraviolet: lessons from colour vision in bees and birds. J. Exp. Biol. 204:2571–2580.

Klahre, U., A. Gurba, K. Hermann, M. Saxenhofer, E. Bossolini, P. M. Guerin, and C. Kuhlemeier. 2011. Pollinator choice in petunia depends on two major genetic loci for floral scent production. Curr. Biol. 21:730–739.

Knapp, S. 2010. On 'various contrivances': pollination, phylogeny and flower form in the Solanaceae. Philos. Trans. R. Soc. B-Biol. Sci. 365:449–460.

Kulcheski, F. R., V. C. Muschner, A. P. Lorenz-Lemke, J. R. Stehmann, S. L. Bonatto, F. M. Salzano, and L. B. Freitas. 2006. Molecular phylogenetic analysis of *Petunia juss*. (Solanaceae). Genetica 126:3–14.

Quattrocchio, F., J. Wing, K. van der Woude, E. Souer, N. de Vetten, J. N. M. Mol, and R. Koes. 1999. Molecular analysis of the *anthocyanin2* gene of petunia and its role in the evolution of flower color. Plant Cell 11:1433–1444.

Raguso, R. A., and M. A. Willis. 2002. Synergy between visual and olfactory cues in nectar feeding by naive hawkmoths, *Manduca sexta*. Anim. Behav. 64:685–695.

Roels, S. A. B., and J. K. Kelly. 2011. Rapid evolution caused by pollinator loss in *Mimulus guttatus*. Evolution 65:2541–2552.

Shimada, Y., M. Ohbayashi, R. Nakano-Shimada, Y. Okinaka, S. Kiyokawa, and Y. Kikuchi. 2001. Genetic engineering of the anthocyanin biosynthetic pathway with flavonoid-3′,5′-hydroxylase: specific switching of the pathway in petunia. Plant Cell Reports 20:456–462.

Stehmann, J. R., A. P. Lorenz-Lemke, L. B. Freitas, and J. Semir. 2009. The genus Petunia. Pp. 1–28 *in* T. Gerats and J. Strommer, eds. Petunia: evolutionary, developmental and physiological genetics. 2nd ed. Springer, New York.

Streisfeld, M. A., and M. D. Rausher. 2009a. Altered trans-regulatory control of gene expression in multiple anthocyanin genes contributes to adaptive flower color evolution in *Mimulus aurantiacus*. Mol. Biol. Evol. 26:433–444.

———. 2009b. Genetic changes contributing to the parallel evolution of red floral pigmentation among Ipomoea species. New Phytol. 183:751–763.

Stuurman, J., M. E. Hoballah, L. Broger, J. Moore, C. Basten, and C. Kuhlemeier. 2004. Dissection of floral pollination syndromes in petunia. Genetics 168:1585–1599.

Tornielli, G., R. Koes, and F. Quattrocchio. 2009. The genetics of flower color. Pp. 269–299 *in* T. Gerats and J. Strommer, eds. Petunia: evolutionary, developmental and physiological genetics. 2nd ed. Springer, New York.

Venail, J., A. Dell'Olivo, and C. Kuhlemeier. 2010. Speciation genes in the genus Petunia. Phil. Trans. R. S. B-Biol. Sci. 365:461–468.

White, R. H., R. D. Stevenson, R. R. Bennett, D. E. Cutler, and W. A. Haber. 1994. Wavelength discrimination and the role of ultraviolet vision in the feeding behavior of hawkmoths. Biotropica 26:427–435.

Wijsman, H. J. W. 1983. On the interrelationships of certain species of Petunia. 2. Experimental data. Crosses between different taxa. Acta Botanica Neerlandica 32:97–107.

Wijsman, H. J. W., and B. M. Vandenberg. 1982. Location of structural genes for glucose phosphate isomerase and for leucyl aminopeptidase on chromosome-VII of Petunia. Theor. Appl. Genet. 63:283–287.

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