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Single Gene–Mediated Shift in Pollinator Attraction in *Petunia* [W]

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Animal-mediated pollination is essential in plant reproductive biology and is often associated with pollination syndromes, sets of floral traits, such as color, scent, shape, or nectar content. Selection by pollinators is often considered a key factor in floral evolution and plant speciation. Our aim is the identification and characterization of the genetic changes that caused the evolution of divergent pollination syndromes in closely related plant species. We focus on *ANTHOCYANIN2* (*AN2*), a well-defined *myb*-type transcription factor that is a major determinant of flower color variation between *Petunia integrifolia* and *Petunia axillaris*. Analysis of sequence variation in *AN2* in wild *P. axillaris* accessions showed that loss-of-function alleles arose at least five times independently. DNA sequence analysis was complemented by functional assays for pollinator preference using genetic introgressions and transgenics. These results show that *AN2* is a major determinant of pollinator attraction. Therefore, changes in a single gene cause a major shift in pollination biology and support the notion that the adaptation of a flowering plant to a new pollinator type may involve a limited number of genes of large effect. Gene identification and analysis of molecular evolution in combination with behavioral and ecological studies can ultimately unravel the evolutionary genetics of pollination syndromes.

INTRODUCTION

Animals play an important role in the sexual reproduction of many flowering plants as vectors for pollen transfer between flowers. Specific sets of floral traits are frequently found to be associated with particular groups of pollinators. Such pollination syndromes have arisen independently in closely related plant species in many angiosperm families (Faegri and van der Pijl, 1979; Proctor et al., 1996; Fenster et al., 2004). The relationship between plants and their pollinators has been compared with a lock and key, where the lock has been fitted to a preexisting key (Grant and Grant, 1965). Although the evidence for adaptation between flowering plant species and their pollinators seems overwhelming (Fenster et al., 2004), aspects of the pollination syndrome concept have been criticized (Herrera, 1996; Waser et al., 1996; Johnson and Steiner, 2000; Thompson, 2001). One may describe plant pollinator systems as a continuum ranging from extreme adaptation of one plant species to a single pollinator, as found in some orchid species (Schiestl et al., 1999), via pollination syndromes, where a plant species is primarily pollinated by a morphologically or behaviorally defined class of pollinators (i.e., long-tongued nocturnal hawk moths) and the plant species features corresponding floral adaptations, to more generalized flower types that are visited by multiple pollinator types.

Many closely related plant species occur in sympatry or parapatry in nature without obvious gene flow among them. However, in many cases where isolation mechanisms, such as pollinator preference, limit interbreeding of sibling species in nature, hand pollination is often easily achieved in the laboratory. Such cross-compatibility provides an opportunity to identify the genetic basis of species-specific adaptations (Bradshaw et al., 1995, 1998; Fishman et al., 2002; Hodges et al., 2002; Stuurman et al., 2004; Galliot et al., 2006), to study their evolutionary history, and to assess the role of adaptive mutations on the genetic isolation between species (Schemske and Bradshaw, 1999; Bradshaw and Schemske, 2003).

Many studies have demonstrated the importance of flower color for pollinator preference (Waser and Price, 1981; Niovi Jones and Reithel, 2001; Bradshaw and Schemske, 2003). Recent studies report that natural variation in flower color can be due to differences both in the activity and expression of biosynthetic enzymes and their transacting regulators (Durbin et al., 2003; Zufall and Rausher, 2004; Schwinn et al., 2006; Whittall et al., 2006).

The genus *Petunia* comprises species with distinct hawk moth and bee pollination syndromes, respectively (Wijsman, 1983; Ando et al., 2001). In combination with the availability of genetic and molecular tools (Gerats and Vandenbussche, 2005), this makes *Petunia* an ideal model system to study the molecular genetic basis of pollination syndromes (Stuurman et al., 2004). *Petunia axillaris* (Lam.; Britton, Sterns, and Poggenb.) has white flowers with long and narrow corolla tubes, emits large amounts of volatiles at night, and contains large volumes of floral nectar (Ando et al., 1995a; Stuurman et al., 2004; Hoballah et al., 2005; Oyama-Okubo et al., 2005). Pollination primarily by nocturnal hawk moths has been observed in the field (Ando et al., 2001).

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Petunia integrifolia (Hook.; Schniz and Thell) has a violet-reddish flower with a short wide corolla tube containing low amounts of nectar and emitting small amounts of volatiles (Ando et al., 1995b; Stuurman et al., 2004; Hoballah et al., 2005); it is primarily visited by solitary bees (Ando et al., 2001). Although *P. axillaris* and *P. integrifolia* can be crossed in the laboratory, no hybrids were detected in sympatric populations (Ando et al., 2001). Recent DNA sequence analyses confirm that the species are closely related and that interspecific relationships are poorly resolved (Ando et al., 2005; Kulcheski et al., 2006). Further work with more polymorphic markers may soon resolve ambiguities and provide a robust phylogeny of the genus.

Quantitative trait loci analysis indicated that pollination syndrome–associated traits, such as morphology, scent, and nectar production, in *P. axillaris* and *P. integrifolia* are based on a few larger and some smaller quantitative trait loci (Stuurman et al., 2004; Galliot et al., 2006). However, none of the genes controlling natural variation in morphology, scent, and nectar production have been identified yet. By contrast, flower color pathways have been worked out in great detail (Holton and Cornish, 1995; Koes et al., 2005), and loci that specify color polymorphism between *P. axillaris* and *P. integrifolia* have been identified (Wijsman, 1983). This makes it feasible to study the genetics, ecology, and evolution of flower color as a pollination syndrome trait.

Anthocyanin biosynthesis of the corolla limb of these species is under the control of transcription factor *ANTHOCYANIN2* (*AN2*) (Wijsman, 1983; Quattrocchio et al., 1999). The absence of anthocyanin pigmentation in the *P. axillaris* corolla limb correlates with loss of *AN2* function through inactivating mutations in the *AN2* coding region (Quattrocchio et al., 1999). This suggests that anthocyanin-colored petals, as in *P. integrifolia*, represent the ancestral and white flowers of the derived phenotype. This trend in flower color evolution was also suggested for angiosperms in general (Harborne, 1982).

Here, we analyze the role of *AN2* in the shift of pollinator preference between *P. integrifolia* and *P. axillaris*. First, we provide a detailed analysis of the molecular evolution of the *AN2* gene using a large collection of freshly collected wild material. Second, by employing genetic substitution and transgenic complementation coupled with pollinator choice experiments, we demonstrate that a color change due to a single-gene polymorphism causes major adaptive shifts in pollinator preference.

RESULTS

Analysis of Molecular Evolution

In a previous study (Quattrocchio et al., 1999), it was shown that in a few *P. hybrida* and three *P. axillaris* accessions, *AN2* alleles are nonfunctional because of nonsense or frameshift mutations in the coding region. To ascertain that these mutations were not due to prolonged cultivation in botanical gardens, we collected new field material. Sequence data were obtained from the entire coding region of *AN2* cDNAs derived from the corollas of 35 *P. axillaris* and *P. integrifolia* accessions (Table 1). Phylogenetic analysis showed that the species alleles fall into two divergent, monophyletic clades (Figure 1A). While all *P. integrifolia* alleles encode putatively functional alleles, all *P. axillaris* alleles carry one of six frameshift or nonsense mutations (Figure 1A) at five different positions. In addition to the three previously found mutations (Quattrocchio et al., 1999), we found three additional and relatively recent loss-of-function mutations (Figure 1A). Because these inactivations were found in freshly collected field material, we conclude that they occurred under natural conditions and were not the result of prolonged cultivation.

In a single sequence (D1 in Figure 1A) and a clade (D2 in Figure 1A), we found at the same site (codon 127) a 4-bp insertion or a single base pair deletion, respectively, which have been previously found in other accessions (Quattrocchio et al., 1999). Short insertions or deletions are typical signatures of imprecise transposon excision. Hence, these two alleles may have been caused by the insertion of the same transposon but two different excision events (Quattrocchio et al., 1999), suggesting that a second deletion polymorphism (Figure 1A, clade E) may also have been caused by a transposon.

The occurrence of multiple independent frameshift or stop codon alleles suggests that loss of color arose multiple times. The alternative scenario, however, is that it is a secondary event following selection for downregulation of the gene through inactivation of the promoter. If promoter inactivation were the primary event, one would expect that all *P. axillaris* alleles have very low expression levels. By contrast, if frameshift or stop codons were the primary event, then expression levels in at least some *P. axillaris* might be comparable to the putatively higher expression levels expected in the anthocyanin-producing flowers of *P. integrifolia*. To test these hypotheses, we performed semiquantitative PCR on cDNA of *P. axillaris* and *P. integrifolia* accessions. The results indicated that *AN2* is expressed at high levels in all *P. integrifolia* accessions, while it is expressed at variable levels in *P. axillaris* accessions (Figure 1B). Six of the 17 *P. axillaris* accessions have an mRNA level that is at least 50% of the average *P. integrifolia* level (Figure 1B). Since the *an2* mutation is recessive, we assume that 50% would be sufficient to give a wild-type color if the gene were otherwise functional. The fact that *AN2* mRNA is present in all *P. axillaris* accessions and in some at comparable levels to *P. integrifolia* suggests that *AN2* was first inactivated by mutations in the coding region and that variation in expression levels is secondary.

Analysis of molecular variation may provide support for past selection on a locus. Overall, Tajima's D (Tajima, 1989) for *P. axillaris* ($D = -1.325$) is only slightly more negative than the value for a linked reference gene ($D = -1.012$) and hence provides no strong evidence for directional selection (see Supplemental Figure 1 online). The McDonald-Kreitman test (McDonald and Kreitman, 1991), capable of detecting directional protein evolution, for the entire coding region of *AN2* in *P. integrifolia* and *P. axillaris* indicated an excess of nonfixed, nonsynonymous mutations ($G = 9.919$; $P = 0.00164$), consistent with relaxed selection pressure in *P. axillaris* after alleles had been fixed for loss-of-function mutations. The distribution of mutations along the coding region of *AN2* is not homogenous with an excess of fixed nonsynonymous mutations in the C terminus of the gene (χ^2 test; $P < 0.01$), while there is no significant difference between both regions for synonymous substitutions (χ^2 test, $P > 0.1$; see Supplemental Table 1 online), suggesting that amino acid substitutions in the C terminus have

Table 1. Taxa, Abbreviations, Collection Localities, and Geographic Data of *Petunia* Accessions Used for This Study

Taxa	Abbreviation	Collection	Locality	GPS Coordinates, Sea Level
Petunia integrifolia inflata	(PintS6)	Vrije Universiteit, Amsterdam		
Petunia integrifolia integrifolia	(PintL)	Botanical Garden, Dresden		
Petunia integrifolia violacea	(PintM)	Botanical Garden, Rostock		
Petunia axillaris parodii	(PaxS7)	Vrije Universiteit, Amsterdam		
Petunia axillaris axillaris	(PaxN)	Botanical Garden, Rostock		
Petunia axillaris axillaris	(PaxQ)	Botanical Garden, Leipzig Northern Argentina		
Petunia axillaris axillaris	(PaxP)	Botanical Garden, Bern		
Petunia axillaris axillaris	(PaxO)	Botanical Garden, Dresden Argentina, provence Buenos Aires		
Petunia integrifolia integrifolia	(PintLC)	Uruguay (2004)	Las Canas	33°09'97.9''S 58°21'40.1''W, 16 m
Petunia integrifolia integrifolia	(PintNB)	Uruguay (2002)	Nuevo Berlin	32°59'01.0''S 58°03'80.7''W, 14 m
Petunia integrifolia integrifolia	(PintPV)	Uruguay (2004)	Puerto Viejo	32°38'14.4'S 58°08'83.7''W, 0 m
Petunia integrifolia integrifolia	(PintR)	Uruguay (2005)	Rivera	31°00'10.1''S 55°37'13.1''W, 224 m
Petunia axillaris axillaris	(PaxC)	Uruguay (2004)	Carmelo	33°56'18.4''S 58°22'13.3''W, 19 m
Petunia axillaris axillaris	(PaxPC)	Uruguay (2002)	Punta Colorada	34°54'15.1''S 55°15'67.4''W, 3 m
Petunia axillaris axillaris	(PaxJI)	Uruguay (2004)	José Ignacio	34°46'35.8''S 54°40'87.2''W, 9m
Petunia axillaris axillaris	(PaxBS)	Uruguay (2004)	Balnearis Solis	34°47'37.1''S 55°23'49.3''W
Petunia axillaris axillaris	(PaxBL)	Uruguay (2002)	Barra Santa Lucia	34°46'59.0''S 56°21'88.7''W, 0 m
Petunia axillaris axillaris	(PaxPE)	Uruguay (2002)	Punta Espinillo	34°50'48.6'' S 56°24'25.8'' W, 10 m
Petunia axillaris axillaris	(PaxPDE)	Uruguay (2002)	Punta dell'Este	34°57'97.5''S 54°57'26.5''W, 16 m
Petunia axillaris axillaris	(PaxB)	Uruguay (2004)	La Barra Km 172	34°52'18.9''S 54°45'02.8''W, 11 m
Petunia axillaris parodii	(PaxGP)	Uruguay (2002)	Gruta del Palacio	33°16'81.5''S 57°08'54.1''W, 94 m
Petunia axillaris axillaris	(PaxSG)	Uruguay (2002)	San Gregorio	33°54'95.4''S 56°45'40.1''W, 98 m
Petunia axillaris axillaris	(PaxF)	Uruguay (2004)	Flores	
Petunia axillaris parodii	(PaxNB)	Uruguay (2002)	Nuevo Berlin	32°59'01.0''S 58°03'80.7''W, 14 m
Petunia axillaris axillaris	(PaxLD)	Uruguay (2002)	Laguna del Diario	34°54'53.3''S 55°00'51.8''W, 12 m
Petunia axillaris parodii	(PaxLC)	Uruguay (2002)	Las Canas	33°09'97.9''S 58°21'40.1''W, 16 m
Petunia axillaris axillaris	(PaxLG)	Uruguay (2005)	Laguna del Garzon	34°48'10.5''S 54°34'56.6''W, 7 m
Petunia axillaris axillaris	(PaxE)	Uruguay (2005)	Ruta 12 Eucalyptus	34°33'95.3''S 55°05'21.5''W, 178 m
Petunia axillaris axillaris	(PaxPUE)	Uruguay (2005)	Pueblo Eden	34°37'59.4''S 55°03'26.7''W, 71 m
Petunia axillaris axillaris	(PaxRO)	Uruguay (2005)	Ruta 9 to Rocha	34°44'16.8''S 54°37'37.9''W, 60 m
Petunia axillaris axillaris	(PaxPA)	Uruguay (2005)	Playa Agraciada	33°48'64.7''S 58°25'58.8''W, 11 m
Petunia axillaris axillaris	(PaxPDA)	Uruguay (2005)	Pan de Azucar	34°46'73.7''S 55°11'07.5''W, 65 m
Petunia axillaris axillaris	(PaxATC)	Uruguay (2005)	Arroyo Terpes Chico	
Petunia axillaris axillaris	(PaxSP)	Uruguay (2005)	Salto del Penitente	34°22'33.2''S 55°03'18.7''W, 2 m
Petunia axillaris axillaris	(PaxEA)	Uruguay (2005)	Estancia Arteaga	
Recombinant inbred line	(WP 117)	Stuurman et al. (2004)		
Recombinant inbred line	(WP 119)	Stuurman et al. (2004)		

A cDNA sequence of *P. integrifolia* (PintDB) was obtained from GenBank (AF146704). Seed material is maintained at the University of Bern and is available upon request.

most likely been fixed before selection of loss-of-function alleles occurred. The fixed amino acid differences, however, did not cause a detectable change in protein function in transient expression assays (Quattrocchio et al., 1999).

Although this analysis of molecular evolution did not provide a clear indication of directional selection, the complete absence of functional alleles argues for selection as a likely factor driving the establishment of loss-of-function alleles in *P. axillaris*. The occurrence of multiple loss-of-function alleles could suggest that the selective advantage of these alleles was not so large that a single adaptive allele would have become fixed throughout the species populations. In geographically structured populations of *Arabidopsis thaliana*, multiple adaptive loss-of-function alleles have been well documented (Johanson et al., 2000; Michaels et al., 2003; Shindo et al., 2005). We did not observe comparable phylogeographic structure of *AN2* alleles in *P. axillaris* (see Supplemental Figure 2 online). Hence, we suggest that loss-of-function alleles with a selective advantage may arise frequently and before selection fixes a single adaptive allele.

Pollinator Observations in the Wild

To determine which pollinators visit the flowers of *P. axillaris* and *P. integrifolia* in the natural habitat, we observed two populations of each species at different locations in Uruguay. *P. axillaris* flowers received some bee and beetle visits during the day and were exclusively visited by hawk moths at night (Figure 2A). *P. integrifolia* flowers did not receive nocturnal visits and were visited by bees and diurnal butterflies during the day (Figure 2B). Thus, in nature, the two species attracted a wider spectrum of visitors in addition to the expected pollinator types. We identified several hawk moth, beetle, and bee species that visited wild *Petunia* species (see Supplemental Table 2 online). The most common bees visiting *P. integrifolia* were *Leioproctus* and

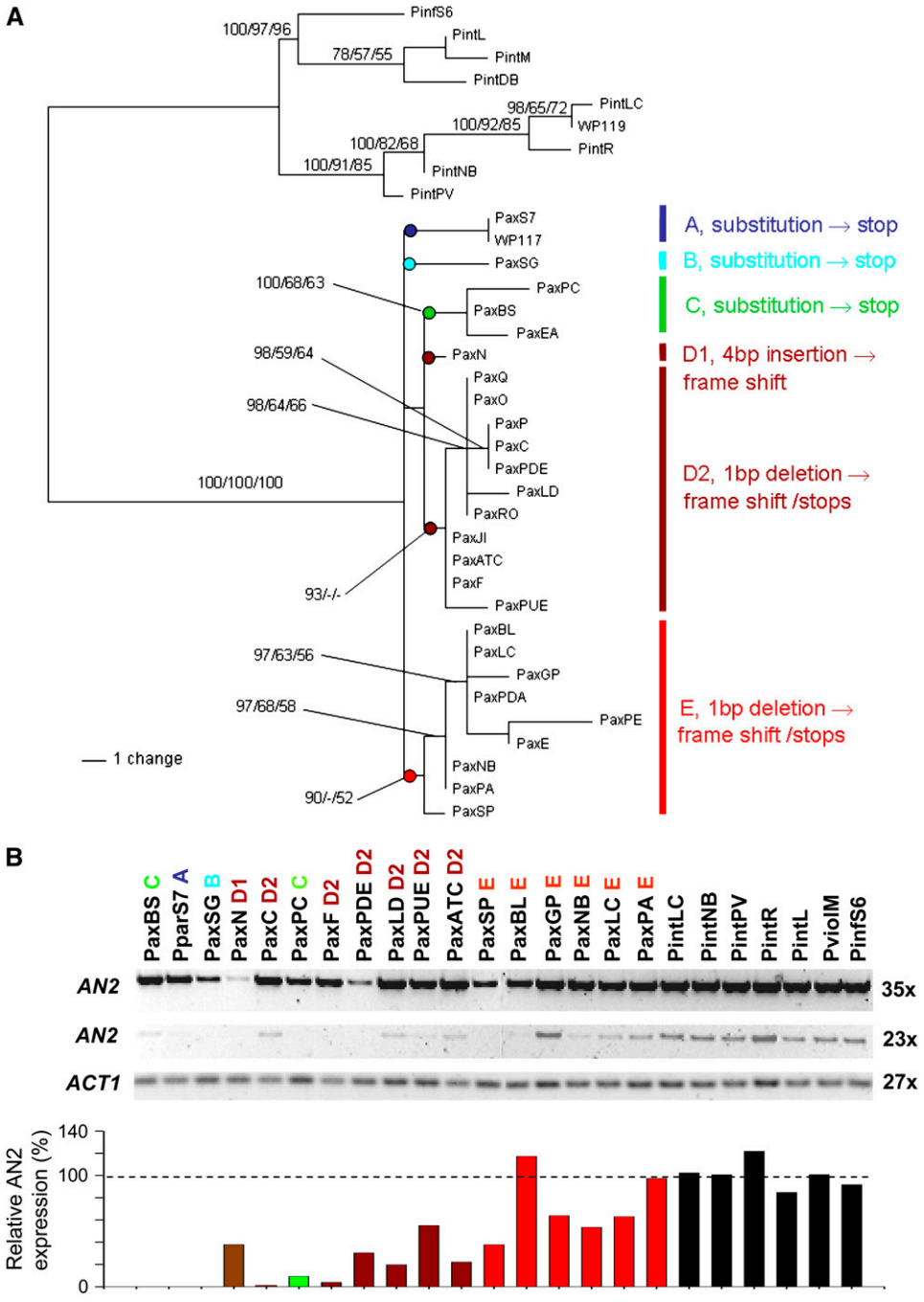

Figure 1. Molecular Analysis of *AN2*.

(A) Bayesian tree of *AN2* cDNA sequences. Five independent loss-of-function mutations (A to E) in *P. axillaris* (Pax) are color coded. Accession abbreviations are given in Table 1. Numbers above branches indicate Bayesian support values/maximum likelihood/maximum parsimony bootstrap support.

(B) *AN2* mRNA is present in all *P. axillaris* and *P. integrifolia* accessions. Semiquantitative RT-PCR of *AN2* in top and middle panels (35 and 23 amplification cycles, respectively) and *ACTIN1* (*ACT1*) control (27 cycles) in bottom panel in *P. axillaris* and *P. integrifolia* petals. All *AN2* expression values were normalized to *ACTIN1*. The average of the *P. integrifolia* levels was taken as 100%.

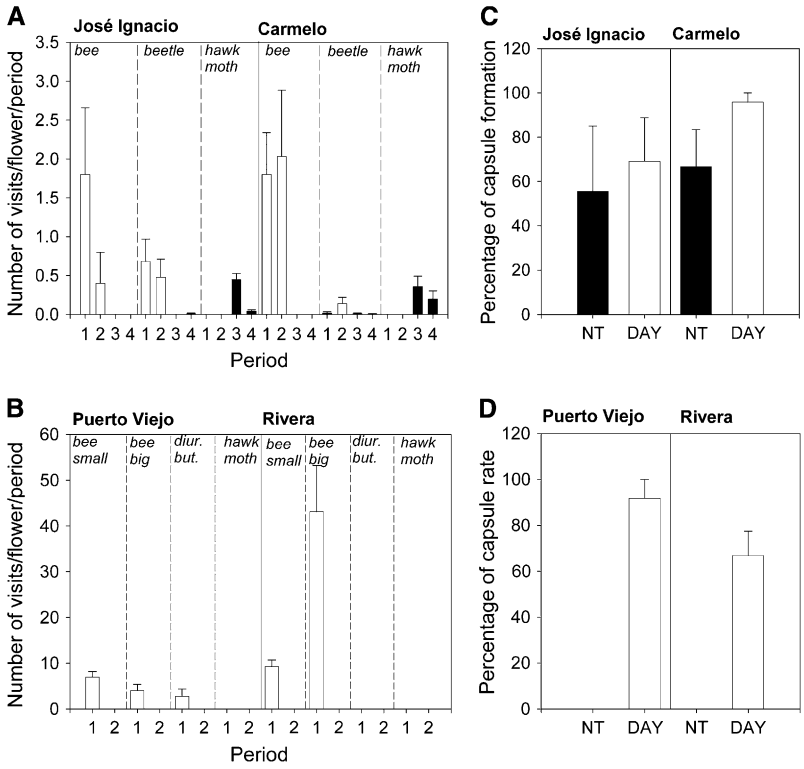

Figure 2. Pollinator Visits to *Petunia* Species in Natural Habitats.

(A) Mean (±SE) number of visits of pollinators to *P. axillaris* per flower per period (1, 8 AM to 12 AM; 2, 2 PM to 6 PM; 3, 8:30 PM to 9:30 PM; 4, 10 PM to 2 AM) in the localities José Ignacio and Carmelo (Uruguay) ($n = 7$).

(B) Mean (±SE) number of visits of pollinators to *P. integrifolia* per flower per period (1, 10 AM to 2 PM; 2, 9 PM to 11 PM) in the localities Puerto Viejo and Rivera (Uruguay) ($n = 7$). Note that *P. integrifolia* was not visited by pollinators at night.

(C) Pollinator exclusion shows that diurnal and nocturnal pollinators effectively pollinate *P. axillaris* in José Ignacio and Carmelo. There was no difference in capsule formation (mean ± SE) in day- or night-pollinated plants (Mann-Whitney test, $Z = -0.711$, $P = 0.477$ for José Ignacio; $Z = -1.38$, $P = 0.167$ for Carmelo, $n = 4$).

(D) *P. integrifolia* is exclusively day-pollinated. Mean (±SE) percentage of capsule formation in Puerto Viejo and Rivera in day-pollinated (no capsule formation) and night-pollinated *P. integrifolia* plants ($n = 4$).

Calliopsis, which were not observed on *P. axillaris* flowers. Pollinator exclusion experiments showed that both diurnal and nocturnal flower visitors can pollinate *P. axillaris* plants (Figure 2C), whereas *P. integrifolia* is exclusively pollinated by diurnal insects (Figure 2D).

The pollination systems found in *P. integrifolia* and *P. axillaris* may be characterized as pollination syndromes, where one plant species is predominately visited and pollinated by a specific type of pollinator but not exclusively so.

AN2 Introgressions Affect Pollinator Choice in the Field

To test the effect of floral anthocyanin pigmentation, controlled by *AN2*, on pollinator preference, we tested pollinator preference for two recombinant inbred lines derived from introgressing *P. axillaris* (Table 1, PaxS7) chromosome segments into a *P. hybrida* W138 background (Stuurman et al., 2004). Introgression line (IL) WP117 containing the inactive *P. axillaris an2*$^-$ allele has very low amounts of anthocyanin (Figure 3A), while WP119 carrying a *P. integrifolia AN2* allele produces anthocyanins (Figure 3B). Both ILs are morphologically intermediate between both species (Figures 3A and 3B), produce virtually no odor (data not shown), and do not differ in nectar volume (Figure 3D) but differ in reflectance (Figure 3C).

In the native environment in José Ignacio, Uruguay, diurnal butterflies and only few other pollinators were observed to visit lines WP119 and WP117. Butterflies preferred the line WP119 versus the line WP117 (Figure 3E; Wilcoxon signed-ranks test, $Z = -2.371$, $P = 0.018$). Under field conditions, only very few visits by hymenopterans were observed, while hawk moths were present but failed to visit either IL, possibly because these lines lack an additional determinant of hawk moth attraction, such as strong scent (Raguso and Willis, 2002).

Field experiments with European hymenopteran pollinators in southern Switzerland showed a clear preference of for WP119 flowers compared with WP117 flowers. Hymenopteran landing rate per flower per hour was significantly higher on WP119 than on WP117 flowers (Figure 3F; Wilcoxon signed ranks test, $Z = -2.023$, $P = 0.043$). Hymenopterans observed landing on these flowers were bumblebees, common honeybees, and

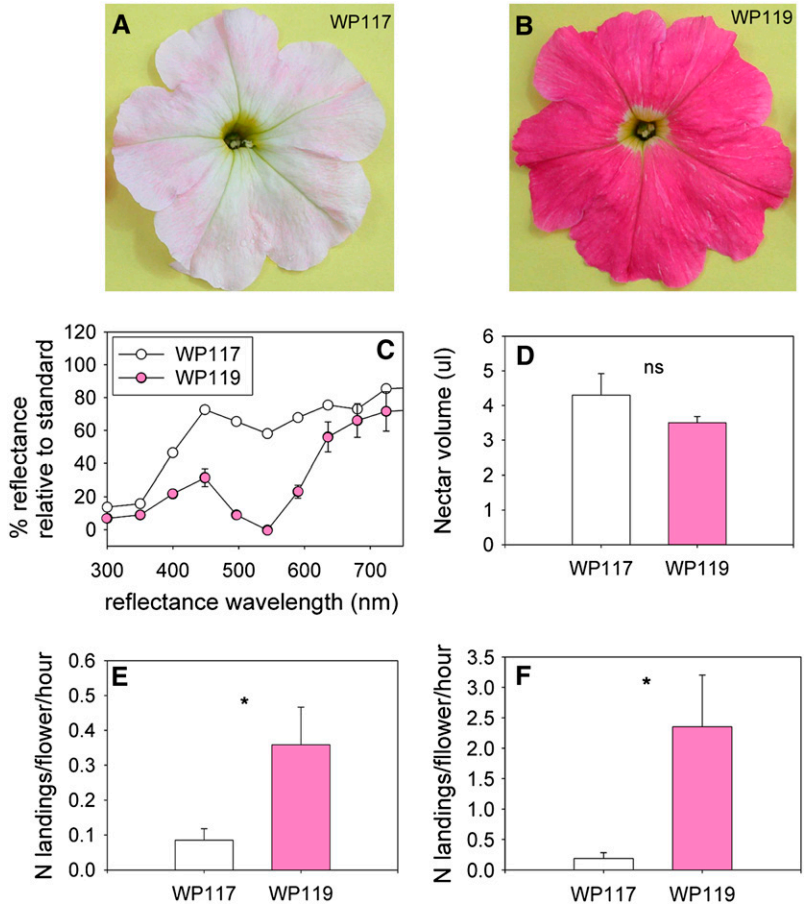

Figure 3. *Petunia* ILs WP117 and WP119 Are Polymorphic for the *AN2* Locus and Affect Pollinator Behavior.

(A) and **(B)** *Petunia* IL WP117 **(A)** carries a nonfunctional *an2* allele and WP119 **(B)** carries an active *AN2* allele (Stuurman et al., 2004).
(C) Mean ± SE of percentage of reflectance relative to a white standard for different wavelengths.
(D) Mean ± SE nectar content does not significantly (ns) differ between lines (Mann-Whitney test, $Z = -0.662$, $P = 0.508$, $n = 6$).
(E) Mean (±SE) number of visits of diurnal butterflies in the wild habitat in José Ignacio (Uruguay) is higher for the line WP119 than for WP117 ($n = 8$).
(F) Mean (±SE) number of visits of hymenopteran (Minusio, Switzerland) is higher for the line WP119 than for WP117 ($n = 5$). Asterisk over bars indicates the significance level of the statistical test ($P = 0.01$).

smaller hymenopterans. Thus, the field experiments show that diurnal pollinators prefer colored flowers. However, hawk moth preference could not be assessed.

Innate Pollinator Preference under Controlled Conditions

While field observations provide insight into the full complexity of pollination under natural conditions, they are less suitable for routinely assessing the effects of large numbers of genetic variants. Therefore, we set up a system with reduced complexity that tests the innate preference of two model pollinators under controlled greenhouse conditions. We first tested the system with the two wild *Petunia* species. In a scent-saturated greenhouse, the hawk moth *Manduca sexta*, a natural pollinator of *P. axillaris* (Figure 4A) in Uruguay, showed significant preference for *P. axillaris* in first-choice feedings (26 versus 3, respectively; binomial test $P < 0.0001$) and number of feedings per time (Figure 4C; Wilcoxon signed-ranks test, $Z = -4.579$, $P < 0.0001$), while the bumblebee *Bombus terrestris*, as a representative hymenopteran pollinator, showed a significantly higher landing rate on *P. integrifolia* than on *P. axillaris* flowers (Figures 4B and 4D; Wilcoxon signed-ranks test, $Z = -3.061$, $P = 0.002$). Thus, the greenhouse system recapitulates important aspects of pollinator preference in the natural habitat and provides a practical means to assay large numbers of genetic variants.

Under the same setup, the naïve *M. sexta* females preferred at first choice the white WP117 over the colored WP119 (26 versus 8, respectively; binomial test $P = 0.004$). The number of feeding events during a period of 5 min by *M. sexta* was also higher for WP117 than for WP119 (Figure 4E; Wilcoxon signed-ranks test, $Z = -4.836$, $P < 0.0001$). Bumblebees displayed the opposite preference. The number of bumblebee landings per plant per flower during 2 h was higher on WP119 than on WP117 (Figure 4F; Wilcoxon signed-ranks test, $Z = -2.044$, $P = 0.041$).

Thus, greenhouse experiments also showed that *Petunia* ILs that are polymorphic for *AN2* are differentially visited by pollinators.

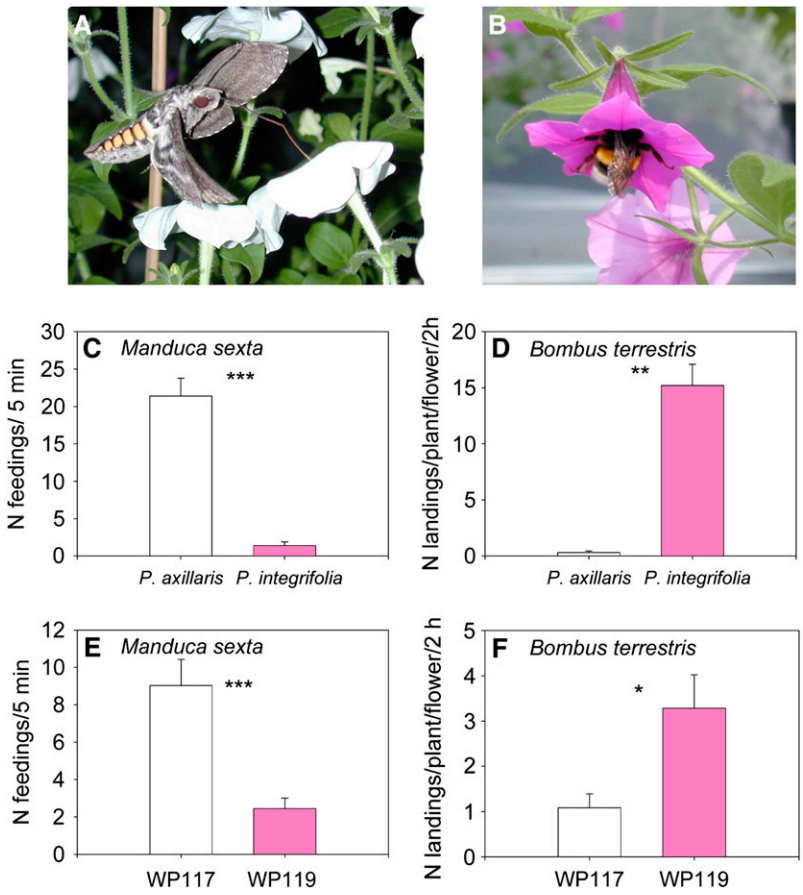

Figure 4. Pollinator Preference for *Petunia* Species under Controlled Greenhouse Conditions.

(A) *M. sexta* feeding on *P. axillaris*.
(B) *B. terrestris* feeding on *P. integrifolia*.
(C) Mean (±SE) number of visits of *M. sexta* naïve females for feeding during 5 min to *P. axillaris* is significantly higher than to *P. integrifolia* flowers ($n = 29$).
(D) Mean (±SE) number of landings per plant per flower in 2 h by *B. terrestris* on *P. integrifolia* is significantly higher than on *P. axillaris* ($n = 12$).
(E) Mean (±SE) number of visits for feeding by *M. sexta* naïve females during 5 min in the greenhouse is significantly higher for WP117 than for WP119 ($n = 34$).
(F) Mean (±SE) number of landings per flower per plant in 2 h of *B. terrestris* in the greenhouse is higher for the line WP119 than for the line WP117 ($n = 12$).
Asterisks over bars in **(C)** to **(F)** indicate the significance level of the statistical tests. *, $P = 0.01$; **, $P = 0.001$; ***, $P = 0.0001$.

AN2 Has a Major Effect on Pollinator Preference

An important limitation of a genetic introgression approach is that the observed effects cannot strictly be ascribed to a single gene. To resolve this issue, we chose a transgenic approach and transformed *P. axillaris* with a functional *AN2 P. integrifolia*–type cDNA and tested pollinator preference in the greenhouse. The fact that species-specific *AN2* alleles differ in their coding region makes *AN2* an ideal gene for a transgenic approach in which the nonfunctional *AN2* allele of *P. axillaris* is complemented by a transgenic allele of *P. integrifolia*. Transformed and nontransformed plants differed in the reflectance of the corolla limb (Figure 5C) but neither in flower morphology (Figures 5A and 5B), emission of volatile compounds (Figure 5D; analysis of variance, $P > 0.2$, $1.762 > F > 0.334$, $n = 11$ wild, $n = 8$ *AN2*-transformed), nectar volume (Figure 5E; analysis of variance, $F = 0.154$, $P = 0.708$, $n = 4$), or nectar sugar concentrations (Figure 5F; Mann-Whitney Test, $-0.866 > Z > -1.732$, $P > 0.083$, $n = 4$). We observed a higher feeding rate in a period of 5 min for wild *P. axillaris* than for *AN2*-transformed plants by the hawk moth *M. sexta* (Figure 5G; Wilcoxon signed-ranks test, $Z = -3.946$, $P < 0.0001$). First choice of these pollinators for flowers of non-transformed plants was significantly higher than for *AN2*-transformed wild plants (30 versus 6, binomial test $P < 0.0001$). When the same choice was offered to *B. terrestris*, a shift of preference in the opposite direction, toward *AN2*-transformed plants, was observed (Figure 5H; Wilcoxon signed-ranks test, $Z = -2.393$, $P = 0.017$). Thus, restoring *AN2* function caused a major shift in pollinator preference.

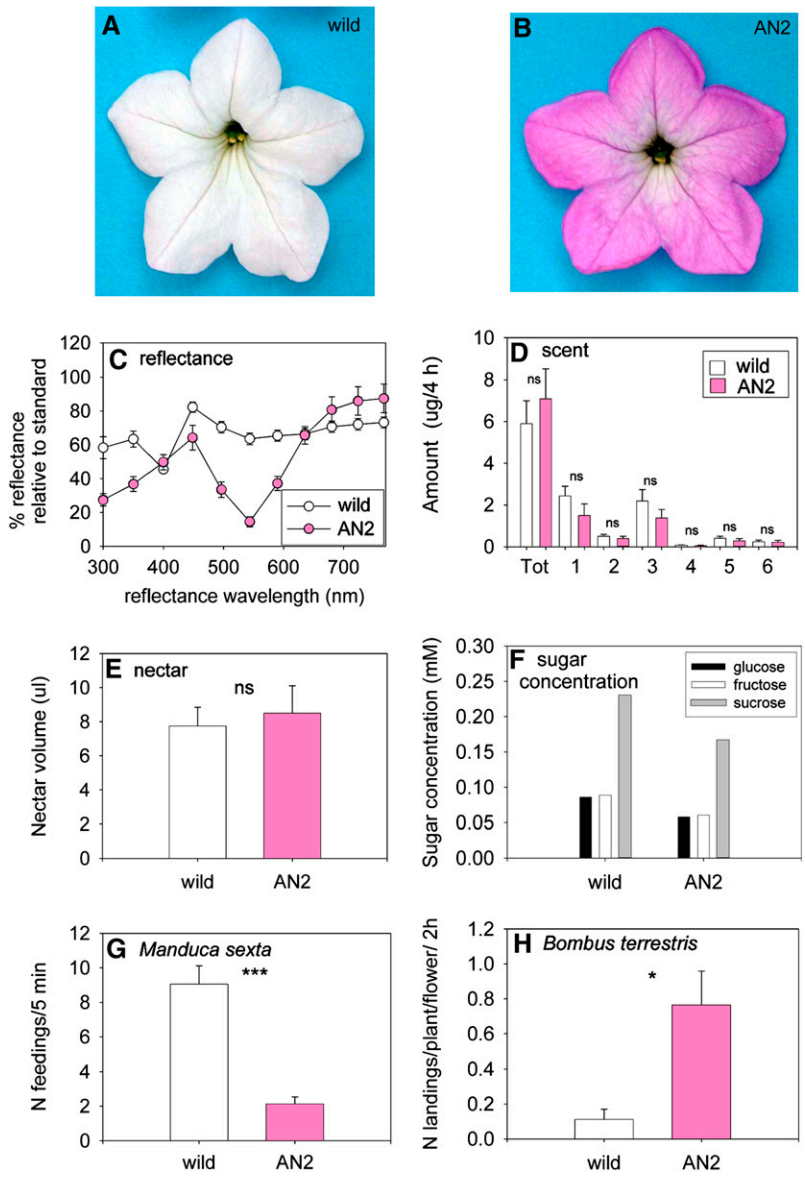

Figure 5. Effect of *AN2* on Pollinator Preference.

(A) and **(B)** Comparison of the wild type **(A)** and *AN2*-transformed *P. axillaris* **(B)** flowers.
(C) Mean ± SE of percentage of reflectance relative to the standard for different wavelengths.
(D) Mean (±SE) production of total volatiles (tot) and amount of single major compounds (1, benzaldehyde; 2, benzyl alcohol; 3, methyl benzoate; 4, methyl salicylate; 5, benzyl benzoate; 6, benzyl salicylate) ($n = 11$ wild; $n = 8$ transformed).
(E) Mean (±SE) nectar amount ($n = 4$). ns, nonsignificant difference between treatments.
(F) Mean (±SE) sugar concentrations ($n = 4$).
(G) Mean (±SE) number of visits for feeding by *M. sexta* is significantly higher for wild *P. axillaris* than for *AN2*-transformed flowers ($n = 36$).
(H) Mean (±SE) number of landings per flower per plant in 2 h of *B. terrestris* is higher on *AN2*-transformed than on wild plants ($n = 12$). Asterisks over bars indicate the significance of the statistical tests (*, P = 0.01; ***, P = 0.0001).

DISCUSSION

A major question in evolutionary biology is how the transition from one adaptive peak (i.e., adaptation to bee pollination) to another adaptive peak (i.e., hawk moth pollination) is achieved, while intermediate phenotypes are expected to have lower fitness than phenotypes at the adaptive peaks (Wright, 1931; Kauffman and Levin, 1987; Whitlock et al., 1995; Whibley et al., 2006). A related question concerns the nature of the genetic changes involved in such transitions: in which order did they occur, what size were the phenotypic effects, and which types of

genes are the targets of selection? The *Petunia* pollination syndromes provide a useful system to address these questions.

Our study of *AN2* provides a first step toward understanding the evolutionary steps involved in the transition between pollination syndromes in the genus *Petunia*. Using introgression and transgenic complementation, we have shown that *AN2* is a major gene affecting both flower color per se and pollinator choice. Variation in *AN2* homologues may also account for flower color variation in more distantly related taxa, such as snapdragon (*Antirrhinum*) species (Schwinn et al., 2006), suggesting that variation in highly specific transcription factors may be a major source of natural phenotypic variation and perhaps the favored target of natural selection in other species as well.

Changes in more general transcription factors, such as *AN1*, which also controls flower color (Spelt et al., 2002), would need to be subtle because of adverse pleiotropic effects. An example of such a scenario would be the *TEOSINTE BRANCHED1* gene, which underwent a subtle change in expression during the domestication of maize (*Zea mays*; Doebley et al., 1997). The case described here is reminiscent of the multiple inactivating mutations in the vernalization genes *FRIGIDA* and *FLOWERING LOCUS C* in geographically structured populations of *Arabidopsis* (Johanson et al., 2000; Le Corre et al., 2002; Michaels et al., 2003; Shindo et al., 2005).

In light of the strong effect of *AN2* alleles on pollinator behavior, it is surprising to find that loss-of-function alleles evolved at least five times independently in *P. axillaris*. Assuming absence of population structure, this suggests that either selection was not very strong, that these alleles arose at very high frequency, or both. This is in line with the marginal signature of selection, as indicated by Tajima's D statistics.

It has been suggested that the evolution of loss-of-function alleles in *AN2* may represent a late step after *Petunia* species had become genetically isolated, as the species-specific alleles share a large number of fixed differences (Quattrocchio et al., 1999). These fixed differences are thought to have evolved before the occurrence of the loss-of-function alleles in *P. axillaris*. While our analysis supports the latter view and the notion that these alleles evolved relatively late, we do not see sufficient evidence for invoking reinforcement (Quattrocchio et al., 1999) (i.e., there is no evidence for an increase of phenotypic differences between the species in zones of sympatry).

Changes in flower color have been shown to reduce interspecific pollen flow and have been implicated in genetic isolation and speciation (Bradshaw et al., 1995, 1998; Clegg and Durbin, 2000; Hodges et al., 2002). The observed changes in pollinator preference associated with *AN2* alleles in *P. axillaris* and *P. integrifolia* are comparable to the ones observed in *Mimulus* species carrying reciprocal introgressions of the *YUP* locus, which affects carotenoid pigmentation (Bradshaw and Schemske, 2003). It will be interesting to know the identity and evolutionary history of the *YUP* locus.

Accepting the hypothesis of a late major shift in flower color in the evolution of *P. axillaris*, a possible scenario to explain the transition from bee to nocturnal hawk moth pollination would be that an intermediate form would have been pollinated predominately by diurnal butterflies or hawk moths, which have an innate preference for colored flowers (Proctor et al., 1996; Kelber, 1997). Subsequent recruitment of nocturnal hawk moths might have involved selection for loss of *AN2* function.

P. axillaris and *P. intergrifolia* display very distinct flower morphologies that conform to the traditional concepts of nocturnal hawk moth and bee pollination syndromes, respectively. However, in the natural habitat, these *Petunia* species are not visited by a single pollinator type but are pollinated by a wider range of pollinator types (Figure 2). Hence, adaptation to a new pollinator type may not leave a strong signature of selection.

The scenario suggested above is obviously speculative. Ultimately, the isolation of additional genes controlling traits such as morphology, scent, and nectar production and the analysis of their molecular evolution in combination with pollinator preference testing of isogenic or transgenic lines both under controlled conditions and in the wild will elucidate the transitions from one pollination syndrome to another.

METHODS

Molecular Analysis of *AN2*

The *AN2* coding region was sequenced from 35 *Petunia* accessions collected in Uruguay or obtained elsewhere (Table 1). cDNA was obtained from total DNase-treated RNA from 2- to 3-d-old flower corollas from plants grown under greenhouse conditions. The primers *AN2* start 5′-GCAGTGAGAACTATACATCATG-3′ and *AN2* stop 5′-TCTTCAATGGTCCCAATTAAC-3′ were used to amplify the *AN2* cDNA with Phusion polymerase (Finnzymes) at 98°C for 30 s and 35 cycles of 98°C for 10 s, 59°C for 30 s, and 72°C for 30 s, followed by 72°C for 5 min. The resulting cDNAs were sequenced by Fasteris. To obtain sequence variation of an additional locus on the same chromosome as *AN2*, we sequenced almost the entire coding region of the *RT* gene encoding rhamnosyl-transferase (Kroon et al., 1994). The primers RT forward 5′-GCTCGCAGTATTAAACAACAG-3′ and RT reverse 5′-CAGCATTTTACAGCCACATTC-3′ were used for amplification and sequencing. PCR conditions were as for *AN2*, but 61°C annealing temperature and an extension time of 40 s semiquantitative PCR on cDNA was performed with RedTaq (Sigma-Aldrich) primers *AN2* qF 5′-GCCACATATAAAAAGAGGGGAC-3′ and *AN2* qR 5′-CAAGAAACATGATTCATTGCCG-3′ with 94°C for 2 min and 23 or 35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 20 s, followed by 72°C for 5 min. For reference, semiquantitative PCR was performed with *ACTIN1* with primers *ACT* F1 5′-TCCATGATTGGAATGGAAGC-3′ and *ACT* R1 5′-GACCCACCACTGAGCACAA-3′ with 94°C for 2 min and 27 and 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 15 s, followed by 72°C for 5 min. All controls without RNA were negative. All *AN2* expression values were normalized to *ACTIN1*. The average of the *Petunia integrifolia* levels was taken as 100%.

Phylogenetic reconstruction was performed using maximum parsimony and maximum likelihood in PAUP version 4.0b 10 (Swofford, 1998) and Bayesian inference in MRBAYES (Huelsenbeck and Ronquist, 2001) under the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985) with a gamma distribution shape parameter $\gamma = 0.1527$ and a transition-transversion ratio of 1.6061, the best-fitting model as determined with MODELTEST 3.7 (Posada and Crandall, 1998). Tajima's D (Tajima, 1989) and the McDonald-Kreitman test (McDonald and Kreitman, 1991) were calculated using DNASP4 (Rozas et al., 2003). For conducting the McDonald-Kreitman test, codons with nonsense or frameshift mutations were removed from the data set.

Petunia axillaris Transformation with *AN2*

The recombinant Ti plasmid pBCaMV, containing a *P. integrifolia*–derived *AN2* cDNA under control of the 35S promoter (Quatrocchio et al., 1993),

was transformed via *Agrobacterium tumefaciens* (strain LBA4404) into leaf discs of PaxS7 (Table 1).

Plants and Pollinators for Choice Experiments

Accessions of *P. axillaris* (PaxS7) and *P. integrifolia* (PintS6) (Table 1) were grown in the greenhouse for behavior experiments. The IL WP117 carries the complete chromosome VI, on which *AN2* is located, from the wild PaxS7 in a *Petunia hybrida* W138 background. IL WP119 possesses half of chromosome VI from the wild species and the other half, including *AN2*, from *P. hybrida* W138. WP119 also contains a small fragment of the PaxS7 genome on chromosome III, which does not influence anthocyanin production.

Flower petal reflectance was measured with a DH2000 Micropack fitted with a UV-VIS-NIR light source (Tecan Rainbow Thermo), and data analysis was performed with the program OOIBase32. Nectar volume, nectar sugar concentration, and odor production were measured as previously described (Stuurman et al., 2004; Hoballah et al., 2005).

Four- to five-day-old, unmated *Manduca sexta* females, obtained as pupae from North Carolina State University Entomology Insectary (Raleigh) and reared under laboratory conditions were used in choice experiments. *Bombus terrestris* were obtained from Andermatt Biocontrol (Switzerland) as a minihive. *B. terrestris* were attracted to *P. integrifolia* flowers in the greenhouse (Figure 4) and could effectively pollinate them: 58.62% seed capsule formation for *B. terrestris*–pollinated flowers ($n = 29$) compared with 56.67% for hand-pollinated flowers ($n = 30$) and 0% for untouched control flowers ($n = 30$). Both *M. sexta* and *B. terrrestris* were naïve when used in choice experiments.

Pollinator Observations in the Wild

Pollinator visitation was observed in natural habitats in two allopatric populations of each species, *P. axillaris* (José Ignacio and Carmelo in January–February, 2004) and *P. integrifolia* (Puerto Viejo and Rivera in January–February, 2005). Pollinators were collected and sent to the Natural History Museum in London for identification.

To assess the effectiveness of diurnal and nocturnal pollinators, some plants were covered during the night (from 8 PM to 8 AM) and some plants during the day (from 8 AM to 8 PM) with insect tents from the time of flower opening until senescence. After flower senescence, the numbers of capsules per plant were counted.

The recombinant inbred lines WP117 and WP119 were tested in the wild in José Ignacio (Uruguay) between January 27 and February 2, 2005. Plants were grown in pots in the greenhouse and then transferred to the field and placed in a semirandomized design. Landings of the pollinators were observed for 4-h periods between 9.45 AM and 2:30 PM during the day ($n = 4$) and for 2-h periods between 9 PM and 12 PM during the night ($n = 5$). The same lines were tested in Minusio, southern Switzerland; observations were performed between 10 AM and 3 PM for 5 h in total. Each test was performed on 6 to 16 plants in a semirandomized design.

Pollinator Preference under Controlled Conditions

Experiments were conducted in a screen cage (144-cm height, 248 × 368 cm) in the middle of a *Petunia* scent–saturated greenhouse. We tested wild *P. integrifolia* versus *P. axillaris* (PaxS7), the recombinant inbred line WP117 versus WP119 (Table 1), and the wild versus the *AN2*-transformed PaxS7. Choice tests were conducted on 8 to 12 plants three to five times in semirandomization. *M. sexta* were allowed to fly and choose freely in the cage for 5 min. We recorded the first choice and the number of total flowers visited for feeding in 5 min. Hawk moths that did not feed in 5 min were discarded and annotated as "no choice"; 19.4 to 26.5% of hawk moths did not choose. For the *B. terrestris* experiments, a minihive was placed at one end of the cage and bumblebee landings on a flower in a period of 2 h were recorded. The experiments were repeated three to four times.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers EF423809 to EF423868. A reference cDNA sequence of *AN2* was obtained from Genbank (accession number AF146704; Table 1).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Tajima's D Results.

Supplemental Figure 2. Phylogeographic Distribution of *P. axillaris AN2* Loss-of-Function Alleles in Uruguay.

Supplemental Table 1. χ^2 Test Results.

Supplemental Table 2. Species and Location of Pollinators Collected from *P. axillaris* and *P. integrifolia* Flowers in the Natural Habitat in Uruguay.

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