Current Biology *21*, 730–739, May 10, 2011 DOI 10.1016/j.cub.2011.03.059

Article

Pollinator Choice in *Petunia* Depends on Two Major Genetic Loci for Floral Scent Production

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Summary

Background: Differences in floral traits, such as petal color, scent, morphology, or nectar quality and quantity, can lead to specific interactions with pollinators and may thereby cause reproductive isolation. *Petunia* provides an attractive model system to study the role of floral characters in reproductive isolation and speciation. The night-active hawkmoth pollinator *Manduca sexta* relies on olfactory cues provided by *Petunia axillaris*. In contrast, *Petunia exserta*, which displays a typical hummingbird pollination syndrome, is devoid of scent. The two species can easily be crossed in the laboratory, which makes it possible to study the genetic basis of the evolution of scent production and the importance of scent for pollinator behavior.

Results: In an F2 population derived from an interspecific cross between *P. axillaris* and *P. exserta*, we identified two quantitative trait loci (QTL) that define the difference between the two species' ability to produce benzenoid volatiles. One of these loci was identified as the MYB transcription factor ODORANT1. Reciprocal introgressions of scent QTL were used for choice experiments under controlled conditions. These experiments demonstrated that the hawkmoth *M. sexta* prefers scented plants and that scent determines choice at a short distance. When exposed to conflicting cues of color versus scent, the insects display no preference, indicating that color and scent are equivalent cues.

Conclusion: Our results show that scent is an important flower trait that defines plant-pollinator interactions at the level of individual plants. The genetic basis underlying such a major phenotypic difference appears to be relatively simple and may enable rapid loss or gain of scent through hybridization.

Introduction

The taxonomy of the angiosperms (flowering plants) has traditionally relied on flower characteristics such as morphology and petal color [1, 2]. These characteristics are also of functional importance because they affect the frequency and specificity of animal visitors. Mutations that alter the shape and color of the corolla may cause shifts in pollinator visitation and thereby lead to reproductive isolation [3]. Thus, genes that specify differences in floral traits are important for the definition of a species. Recent genetic analyses have shown that the substitution of a single locus or even a single gene can have a major influence on pollinator specificity between closely related species [4–6]. However, pollinator preference is not specified by a single trait but by suites of floral characters, referred to as pollination syndromes [7, 8]. In addition to color and morphology, fragrance and nectar quality and quantity are important cues [7, 9].

When modification of a single locus can cause a shift in pollinator visitation, the question arises of what the function of multiple cues might be. Are they purely redundant, qualitatively different, and/or do they act synergistically? The role of scent can be surprisingly complex. It can be a highly specific cue, as in sexually deceptive orchids [10], but also a more generalized long-distance cue that acts synergistically with visual cues [11]. Fragrance blends may also contain components that limit foraging time, and thereby promote outcrossing [12], or play a role in defense against herbivores [13, 14].

The genus *Petunia* is spread across southern South America and comprises species with distinct pollination syndromes [8, 15]. The ancestral syndrome, typified by *Petunia integrifolia*, is bee pollination. The violet-colored *P. integrifolia* flowers have short wide corolla tubes and produce small quantities of nectar and little or no fragrance. *Petunia axillaris* is pollinated by nocturnal hawkmoths, including *Manduca sexta*, and presents white flowers with long, narrow tubes. It produces copious amounts of volatiles and nectar [15–18]. *Petunia exserta* displays a typical hummingbird syndrome [19] with bright red corolla, exserted sexual organs, no scent, and large amounts of nectar [15, 19, 20] (Figure 1A). *P. axillaris* and *P. exserta* are closely related, and their exact phylogenetic position remains uncertain. On the basis of biogeographic data, it is assumed that *P. exserta* is derived from *P. axillaris*. Although all species readily hybridize in the laboratory, natural hybrids between *P. axillaris* and *P. integrifolia* have never been observed in nature [21]. In contrast, plants with intermediate flower morphologies are present in sympatric populations of *P. axillaris* and *P. exserta*, and interspecific hybridization is considered a major threat to the survival of the very rare *P. exserta* [22].

Biosynthetic pathways of scent production and structural and regulatory genes have been well studied in *Petunia* [23–27]. Benzenoids are the main volatiles, but smaller amounts of fatty acid derivatives are also present [27, 28]. Benzaldehyde and methylbenzoate could be measured in all *P. axillaris*, whereas other benzenoids were present in some but not all accessions [27–29]. Furthermore, methylbenzoate, benzyl alcohol and benzaldehyde were found to elicit strong responses from a native hawkmoth pollinator, *M. sexta,* in electroantennogram assays [28].

Here we report on the genetic dissection of fragrance in crosses between *P. axillaris* and *P. exserta* (Figure 1A). We identified QTL on chromosomes II and VII and established their epistatic relationship. Using a repetitive backcross scheme, we constructed near isogenic lines (NIL) that differed only in petal color and/or fragrance. These lines were used in choice experiments to analyze the preference of *M. sexta* under defined conditions.

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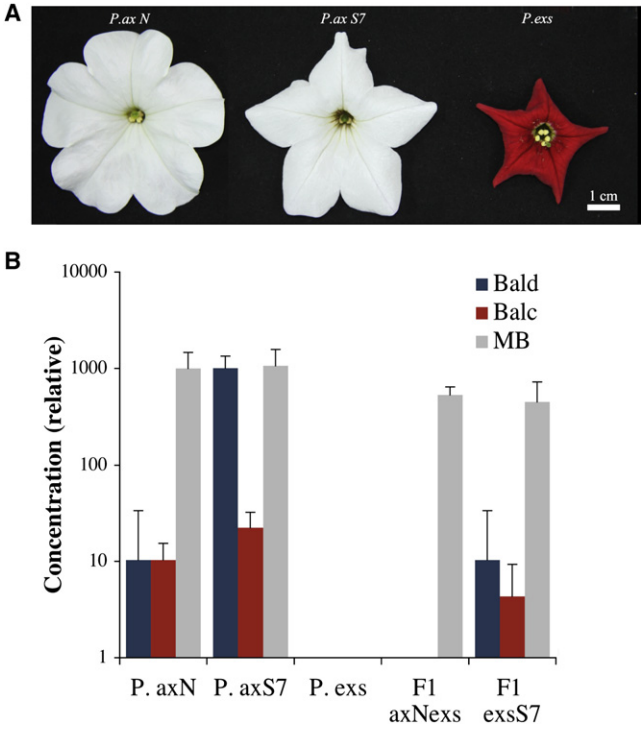

Figure 1. Plants Used in This Study

(A) Flowers of *Petunia* species used in this study: *P. axillaris* N (*P. axN*) *P. axillaris* S7 (*P. axS7*). and *P. exserta* (*P. exs*). The scale bar represents 1 cm.
(B) Scent production by *P. axillaris* and *P. exserta* species and their F1s with PTR-MS. The following abbreviations are used: Bald: benzaldehyde, Balc: benzyl alcohol, MB: methyl benzoate. Error bars indicate standard deviations. See also Figure S1.

Results

Measurement of Volatiles

We used proton-transfer-reaction mass spectrometry (PTR-MS) for fast high-throughput measurements of volatiles from the floral headspace. In a comparison with classical gas chromatography (GC), we found the same benzenoid compounds albeit with different relative proportions. A detailed comparison of the two methods can be found in the Supplemental Information, available online (Figure S1). The two *P. axillaris* accessions used in this study, *P axillaris* N and *P. axillaris parodii* S7 (hereafter referred to as *P. axillaris* N and *P. axillaris* S7) produced copious amounts of methylbenzoate and varying levels of benzaldehyde and benzylalcohol (Figure 1B). *P. axillaris* S7 produced no isoeugenol as reported [30] (Figure S1). *P. exserta* flowers produced no detectable volatile compounds by either PTR-MS or GC measurements (Figure 1B and Figure S1). This is consistent with the lack of flower volatiles in other hummingbird-pollinated species [31].

Scent bouquets of wild accessions freshly collected from a wide geographical distribution in South America (Figure S1A) varied substantially, but all tested plants produced large amounts of methylbenzoate. Thus, *P. axillaris* N and *P. axillaris* S7 provide representative examples of the species' volatile cocktail. We focused our attention on methylbenzoate because this compound was reliably detected among the predominating volatiles in all *P. axillaris* species and is a main chemical stimulus eliciting electroantennogram responses in *M. sexta* (Figure S1; see also [28]).

Interspecific Crosses between Scented and Nonscented Species

We used an F2 population of a cross between *P. axillaris* N and *P. exserta* for a quantitative trait locus (QTL) analysis. PTR-MS and GC-measurements showed that F1 plants produced slightly lower amounts of volatiles compared to the *P. axillaris* parent (Figure 1B and Figure S1B). This suggests the presence of (semi)dominant loci for scent production in the *P. axillaris* genome.

Methylbenzoate emission was measured from flowers of the *P. axillaris* N × *P. exserta* F2 population (Figure 2A). Of 203 F2 plants, 51 did not produce any detectable scent, whereas the rest of the plants showed values that ranged from very low scent to the levels of *P. axillaris* flowers. The simplest explanation for the segregation data is a single major locus controlling scent that is epistatic over one or more additional loci of quantitative effect.

QTL Analysis and Trait Introgression

For mapping purposes simple sequence repeat (SSR) [32] and cleaved amplified polymorphic sequence (CAPS) markers were developed to cover the seven chromosomes of *Petunia* in intervals of 5–10 centiMorgan (Figure 2B). QTL analysis (Figure 2C) revealed two major loci responsible for the difference of scent production between *P. axillaris N* and *P. exserta*: one on chromosome II explaining about 40% of the parental difference and one on chromosome VII that explains almost 20%. Both loci were supported by highly significant p values and LOD scores (see Experimental Procedures; Figure S2A). A minor QTL was located on chromosome III.

To further characterize the major QTL, we backcrossed informative F2 lines and bred introgression lines that differ exclusively in scent production by using a combination of phenotypic and marker-assisted selection. However, we noticed that none of the red F2 plants produced volatiles, presumably because the scent QTL on chromosome II is linked to a locus important for color production. This locus does not correspond to AN2 or other known genes involved in anthocyanin production and its identity is presently unknown. In contrast to *P. axilllaris* N, plants of a cross with *P. axillaris* S7 produced more anthocyanins in flower petals and we were able to breed for fully red, scented plants. We therefore also analyzed 134 plants of a F2 population between *P. exserta* and *P. axillaris* S7, and identified QTL on chromosomes II and VII, (Figures S2B and S2C), corroborating the data obtained for the *P. axillaris* N x *P. exserta* population. Therefore we produced near isogenic lines (for details see Figure S3) by introgressing loci of *P. exserta* into the *P. axillaris* S7 genome to generate a scentless *P. axillaris*. Conversely, we introgressed loci from *P. axillaris* S7 into the *P. exserta* genomic background to end up with scented plants that resembled *P. exserta* in all other traits, i.e., a scented *P. exserta*.

Scentless *P. axillaris* plants were bred by selecting F2 plants that were most similar to *P. axillaris* but did not produce scent. After genotyping, suitable candidates were selfed or backcrossed to *P. axillaris*. In the eighth generation plants that were nonscented but otherwise indistinguishable from *P. axillaris* were obtained (Figure 4). Fine mapping of sibling plants that segregated 3:1 for scent indicated that the introgressed interval on chromosome II corresponds to less than 1 centiMorgan (Figures 2D and 4C). Scented and nonscented

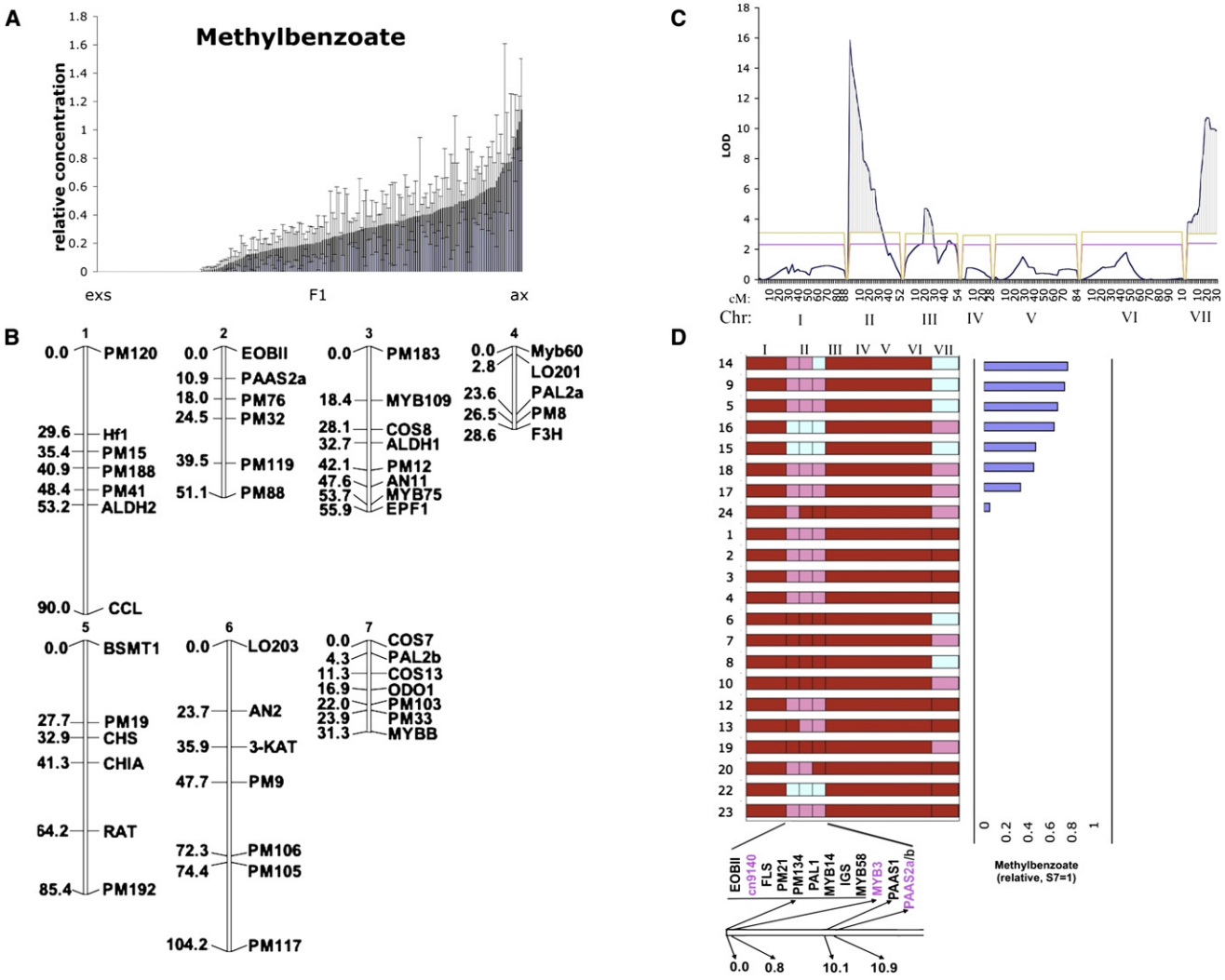

Figure 2. Scent Measurement and QTL Analysis in a F2 Population of *P. axillaris* and *P. exserta*

(A) Scent measurements of F2 progeny of a cross between *P. axillaris N* and *P. exserta* plants. Measurements were normalized to the average level of each session (see Experimental Procedures). Error bars are standard deviations. Values for parents (axN, exs) and F1 plants are indicated.

(B) Genetic map of F2 *P. axillaris* N × *P. exserta* population used for QTL mapping. CAPS and SSR markers were placed with approximately 5–10 centiMorgan distances between markers.

(C) Interval mapping for the seven chromosomes of *Petunia* as calculated by the Qgene program (simple interval mapping). The blue line is the LOD score; the horizontal lines correspond to the threshold for highly significant (p = 0.01, yellow) and significant (p = 0.05, pink) LODs determined by resampling. Roman numbers below the graph are chromosome numbers; mapping distances per chromosome are given in centiMorgan. See also Figure S2.

(D) Measurement of scent production of a representative segregating population of red-flowered, scented plants (population S in Figure S3; methylbenzoate is given as percent of emission of the *P. axillaris* S7 parent; error bars indicate standard deviations). Roman numbers represent chromosomes; red boxes represent homozygous *P. exserta* alleles, pink are heterozygous, and blue are homozygous *P. axillaris* alleles. Additional markers are shown for the area of the QTL on chromosome II. Purple markers were used for mapping the segregating population. See also Figure S4.

sibling plants of this generation were selected for further characterization and behavioral experiments and named white nonscented line (WNS) and white scented line (WS).

To test for the effect of the QTL on chromosome VII, introgression lines that contained only chromosome VII of *P. exserta* in a background of *P. axillaris* were bred. These plants produced less than half the scent of the parent (Figure 3D). This shows that the QTL on chromosome VII makes a substantial contribution to scent quantity, but it is not absolutely required for scent production per se (see also below).

To breed for a scented *P. exserta* we selected scented individuals among the F2 plants that were phenotypically closest to *P. exserta* and backcrossed them repeatedly to *P. exserta*. Mapping of several segregating populations showed that plants homozygous for *P. exserta* chromosome II did not produce scent; plants that were homozygous for *P. exserta* on chromosome VII produced less than 30% and in most cases no scent. By crossing two nonscented lines that were heterozygous only for parts of chromosome II and VII, respectively, we obtained seven scented out of 24 plants (Figure S4). This is consistent with two dominant *P. axillaris* loci, on chromosome II and chromosome VII, being sufficient for scent production. Red flowering introgression lines that contained the relevant parts of chromosomes II and VII from *P. axillaris* in a *P. exserta* background were selected for further experiments. Siblings of a segregating population were

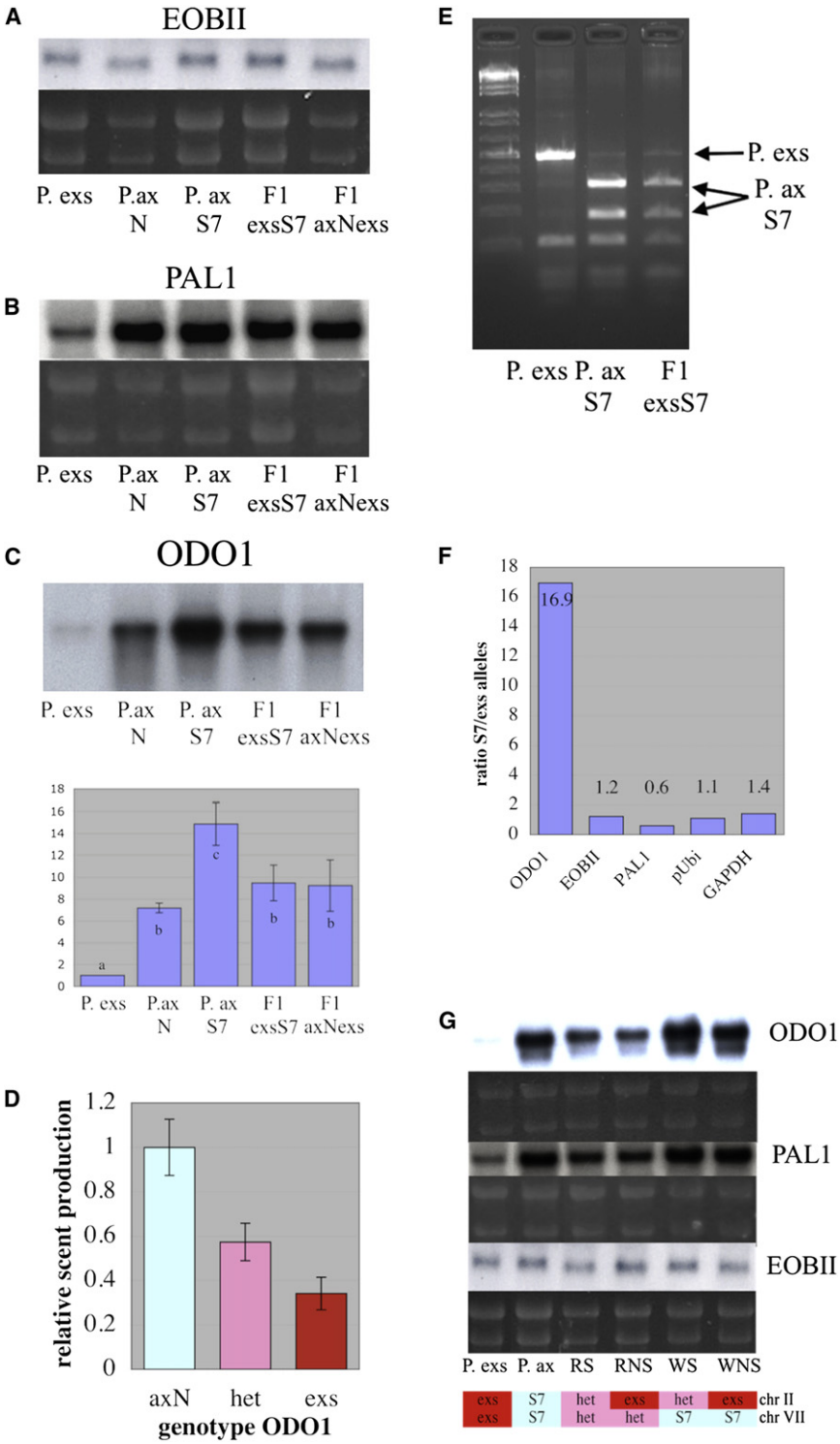

Figure 3. Correlation of Candidate Gene Expression with Scent Production

(A) *EOBII* expression of parent accessions and their F1s shown by gel blots of RNA isolated from petals. The following abbreviations are used: P. axN, *P. axillaris N*; P. axS7, *P. axillaris* S7; P. exs, *P. exserta*; F1 exsS7, F1 between *P. exserta* and *P. axillaris* S7; F1axs Nexs, F1 between *P. axillaris* N and *P. exserta*. The ethidium bromide staining of the RNA gel is shown below the blot.

(B) PAL1 expression in parent accessions and their F1s. Labeling of lanes as in (A).

(C) ODO1 expression of parent accessions and their F1s. Labeling of lanes as in (A). A quantification of four independent gel blots is shown in the lower panel. Expression values were normalized to the expression in *P. exserta* (= 1); error bars indicate standard deviations and the same letters in the bars denote expression levels that are not significantly different (Tuckey's honestly significant difference test, alpha = 0.05). See also Figure S5.

(D) Correlation of *ODO1* genotype and scent production. Bulk segregant analysis for a population segregating for chromosome VII in a *P. axillaris* background. Colors represent the genotype of *ODO1* indicated on the x axis. Measurements of methylbenzoate were pooled from a segregating population. Error bars indicate standard deviations.

(E) Allele-specific RT-PCR-CAPS restriction digests of cDNA from parent and F1 plants. Fragment sizes expected for the different genotypes are indicated. Labels correspond to those in (A).

(F) Quantification of transcripts in an F1 between *P. exserta* and *P. axillaris* S7 by Illumina transcript profiling of RNA derived from petals. For labels see (A).

(G) Northern blot for *ODO1*, *PAL1*, and *EOBII* expression with RNA from parents and introgression lines. For labels see (A). Introgression lines RS, RNS, WS, and WNS (see Figure S3). The genetic constitution at the loci on chromosomes II and VII is indicated at the bottom.

named red scented line (RS) and red nonscented line (RNS; Figure S3).

Chromosome II Candidate Genes

We identified polymorphisms in genes encoding proteins with a role in scent production and checked whether any of them mapped to the QTL. Because MYB and bHLH transcription factors were shown to be crucial in the regulation of other phenylpropanoid compounds [33–36], we developed markers for all available genes that may play a role in flower gene expression. A total of 29 genes could be excluded from the list because they did not map to the introgressed chromosome segments in the most advanced crosses (for details see http://www.ips.unibe.ch/deve/caps/index.html and Supplemental Information).

Of the genes that were mapped to the top of chromosome II (Figure 2D), *PAL1* and *EOBII* were of particular interest because they were retained in the advanced introgression lines. EOBII is a MYB transcription factor that regulates another MYB factor, *ODO1* [26]. Low EOBII expression or inactive protein in *P. exserta* could explain lower levels of ODO1 and PAL in this species. We found only silent polymorphisms in the EOBII coding region and no difference in expression between *P. exserta* and the scented species (Figure 3A). We therefore conclude that it is unlikely that EOBII underlies the large-effect QTL on chromosome II.

The other candidate gene on chromosome II, *PAL1*, encodes an enzyme that initiates the flow of substrate into the phenylpropanoid pathway. *PAL1* expression levels are lower but not absent in *P. exserta*, making PAL1 an implausible candidate to explain the complete absence of scent production. Sequence analysis showed that only one nucleotide change (G882A) in *P. exserta* leads to an amino acid change (D235N) in a poorly conserved region of the protein.

Chromosome VII Candidate Gene *ODO1*

The MYB protein ODO1 is a transcriptional regulator of several enzymes involved in scent production [27]. Because it was mapped to chromosome VII, *ODO1* may be the gene responsible for the QTL on this chromosome. We therefore investigated the expression of this gene and its correlation with scent production in more detail by using wild accessions and our interspecific crosses.

ODO1 expression in wild accessions of *Petunia* correlated with scent production (Figure S5). High levels were found in *P. axillaris* plants that emit high amounts of volatiles, whereas *P. integrifolia* and *P. exserta*, which do not produce these compounds, expressed background levels of ODO1 mRNA. Low levels were detected in *P. inflata* S6, in which only benzaldehyde was found [28]. Next we determined *ODO1* expression levels in the parent species and their F1 crosses (Figure 3C). As expected, both the *P. axillaris* lines and the F1s showed high expression, whereas *P. exserta* barely expressed any *ODO1*. Quantification showed an approximately 10-fold higher expression in the scented than in the nonscented lines (Figure 3C, lower).

We sequenced *ODO1*, including a 1.2 kb promoter fragment, from several species. The *P. exserta* promoter showed no large deletions or insertions and few nucleotide changes compared to the *P. axillaris* alleles. The *P. exserta* coding region showed one alteration leading to an amino acid change (V235A), which was not found in *P. axillaris* alleles. Because this is a conservative change, it is unlikely to have a major effect (see also below).

To quantify the contribution to scent production of the *P. exserta* allele of *ODO1*, we produced a population where only the *ODO1*-containing genome segment segregated in a *P. axillaris* background. Plants homozygous for *P. exserta ODO1* produced lower amounts of volatiles than plants homozygous for *P. axillaris*, and heterozygotes produced intermediate levels (Figure 3D). The reason for this finding might be that the *ODO1* allele of *P. exserta*, although functional, produces fewer transcripts than the *P. axillaris* allele.

F1 hybrids provide a useful tool to distinguish between differences in *cis*- or *trans*-activation of species-specific alleles of a gene [37]. To do so, we PCR amplified ODO1 cDNAs from the parents and the F1s and used allele-specific restriction sites to determine the relative expression levels (Figure 3E). F1 plants produced substantially more ODO1 transcript from the *P. axillaris* parent than from *P. exserta*, indicating that differences in promoter activity exist between the two species. This result was confirmed by transcript profiling of an F1 plant (for details see Experimental Procedures). ODO1 transcripts derived from the *P. axillaris* S7 allele were 17 times more abundant than the *P. exserta* allele (Figure 3F), which reflects the situation in the parents determined by RNA gel blot (Figure 3C). This experiment also confirmed that *EOBII* and *PAL1* were equally expressed from the two alleles.

Because the genetic data on scent production indicated that the QTL on chromosome II is epistatic over chromosome VII, we asked whether the expression of *ODO1* depends on chromosome II. We therefore checked for *ODO1* expression in the introgression lines that differed in the chromosome II genotype (Figure 3G). The results show that whenever a copy of *P. axillaris* chromosome VII is present, ODO1 levels are significantly elevated relative to *P. exserta*, irrespective of the origin of the chromosome II locus. Therefore, the chromosome II QTL does not affect ODO1 transcript accumulation. *PAL1* expression levels correlate with *ODO1* levels, which is expected because *PAL1* has been shown to be regulated by ODO1 [27]. EOBII mRNA levels were the same in all plants.

M. sexta Prefer Scented Plants, Be They Red or White

In order to use NILs for behavioral experiments with *M. sexta*, we had to ascertain that the lines used differed from their siblings or from the recurrent parent only regarding the odor trait. We measured flavonol levels, anthocyanin levels, limb surface, and nectar content in the RS, RNS, WNS, and WS siblings (Figures 4A and 4B). None of these traits were significantly different between siblings or when compared to the recurrent parent, with the exception that the red introgression lines may contain slightly lower levels of anthocyanin (Figure 4A). Scent segregates as simple dominant marker in both backgrounds (Figures 4C and 4D). We therefore conclude that the only parameter differing between the sibling plants that we tested in insect choice tests was the amount of volatiles produced by the flowers.

Night-active hawkmoths orient to flowers they feed on by using volatiles [38–40]. Studies in which multiple cues were disentangled by physical manipulations suggest complex interactions between olfaction and other stimuli [11, 41, 42]. To clarify the effects of color and scent on pollination frequency in the context of intact plants, we performed wind tunnel experiments with *M. sexta*, a natural pollinator of *P. axillaris* [18] (Figure 5A). When the two wild species, *P. exserta* and *P. axillaris,* were placed 40 cm apart in a simple dual choice experiment that allows the moths to see the plants, only few moths visited *P. exserta*, as expected (exact binomial test, $p < 0.05$; Figure 5B). After they took off, the moths first initiated casting across the wind tunnel before flying upwind. In this setup the hawkmoths may be guided more by the color than by the scent. To test for only the scent component, we positioned plants of both species upwind behind a screen. The moths reached the upwind end of the wind tunnel while flying in the plume of odor of *P. axillaris* and then initiated local casting just downwind of *P. axillaris* at the same height as the scented flowers and 56% of them made contact on the screen with their forelegs. However, they flew slower when no visual cues were present, and they were more likely to fly back downwind and initiate a new upwind flight in the plume. Only few moths visited *P. exserta* first and none of these persisted in flight to this species as they rapidly shifted to the scented side. As a control, we tested behavior in the absence of both visual and olfactory stimuli. When *P. exserta* and the RNS introgression line were placed behind the screen, only a few moths were activated and none showed upwind flight (16 moths tested).

To disentangle visual from volatile stimuli, we used the introgression lines that are indistinguishable by eye. Plants were placed at the upwind end of the wind tunnel in front of the screen. When nonscented and scented *P. axillaris*-like plants were tested (WS versus WNS), moths clearly preferred the scented plants (exact binomial test, $p < 0.001$; Figure 5B). When nonscented and scented *P. exserta*-like plants (RNS

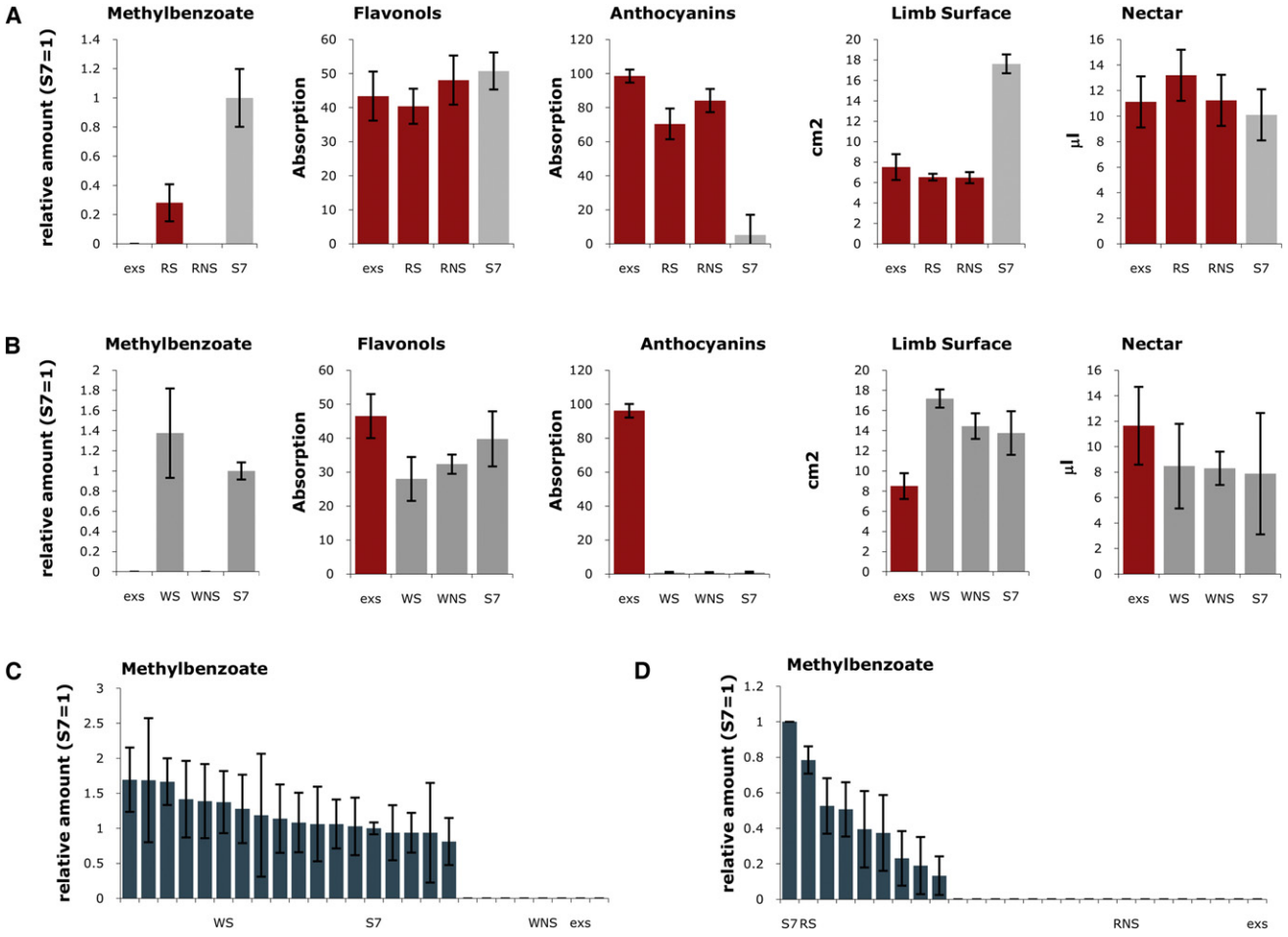

Figure 4. Measurement of Flower Traits of Introgression Lines

(A) Scent, surface, nectar, flavonol, and anthocyanin measurements for the red introgression lines. For more details, see Experimental Procedures. The following abbreviations are used: exs, *P. exserta*; S7, *P. axillaris* S7.

(B) Scent surface, nectar, flavonol, and anthocyanin measurements of white introgression lines. Measurements were done as for (A).

(C) Segregation of scent production in the population used for isolating WS and WNS introgression lines. Scent is shown as a relative value, defining the *P. axillaris* S7 parent as 1.

(D) Segregation of scent production in the population used for isolating the RS and RNSintrogression lines. Scent is shown as a relative value, defining the *P. axillaris* S7 parent as 1.

Error bars for all panels indicate standard deviations.

versus RS) were tested, the moths were also attracted by the scented line (exact binomial test, $p < 0.01$; Figure 5B*)*. Similar results were obtained when scented RS plants were compared to the parent *P. exserta*. However, even though the moths invariably visited the scented flowers at first in these experiments, they often visited the nonscented plant subsequently.

When we exposed the moths to conflicting cues, that is, a nonscented white *P. axillaris*-like plant (WNS) and a scented red *P. exserta*-like plant (RS), the moths flew in the plume of odor, but on arriving at the plants they usually made lateral casting from one plant to the other before making any choice. The moths visited both plants equally as a first choice (exact binomial test, $p > 0.99$; Figure 5B). This means that there was no distinct choice of color over scent by the moths for the first choice but they ended up feeding preferentially on the scented flower (40% RS, 20% WNS).

In all the choice experiments where flower visitation was allowed, 85% of the moths visited the plants, but only 52% of these moths initiated proboscis extension. Nevertheless, the 53 that fed did so preferentially on the scented flowers (82%; exact binomial test, $p < 0.001$).

These experiments clearly show that volatile production affects not only the pollinator's choice over distance, i.e., to find a population of plants, but also determines which individual is pollinated first. We have observed that moths tend to visit plants without scent production as a second choice once in the vicinity.

Discussion

In this work we studied two closely related sister species, *P. axillaris* and *P. exserta*, which differ in scent production and petal color. *P. axillaris* is white and heavily scented, whereas the intensely red flowers of *P. exserta* are devoid of scent. We detected two QTL for scent production of major effect on chromosomes II and VII, as well as a smaller QTL on chromosome III. The variability in scent levels observed in individual F2 plants (Figure 2A) suggests that additional minor

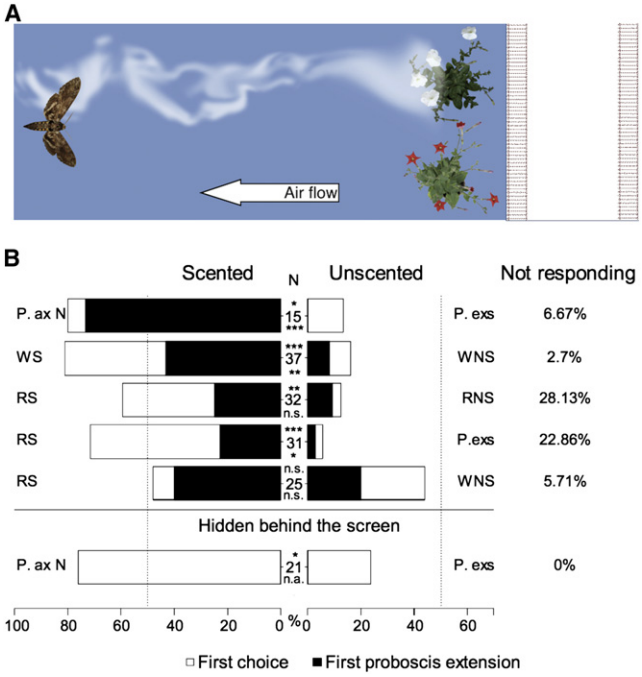

Figure 5. Moths Prefer Scented Flowers, Be They Red or White

(A) Setup of wind tunnel, where pure, standardized air (see Experimental Procedures) enters from the right in a laminar flow. Plants are placed between the two honey comb screens at the upwind end of the wind tunnel (brown, hatched bars) during the blind experiments.

(B) Choice experiments investigating preferences by the moth *M. sexta* for scented flowers. For each experiment, the proportion of the moths that made their first choice (white bars) and first proboscis extension (black bars) on scented plants (left) is plotted against unscented plant (right bars). N is the number of moths tested in each experiment and asterisks above and below N represent, respectively, probability levels on first choice and first proboscis extension by *M. sexta* on plants(*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; exact binomial test). Moths that made no choice (not responding) either did not reach the plants or flew to the downwind end of the wind tunnel or to its floor.

QTL have escaped detection. Introgression of the *P. exserta* chromosome II locus into *P. axillaris* abolishes scent production, signifying that this locus is absolutely required. The combined data indicate that the locus on chromosome VII has a quantitative effect on scent production. We investigated known and putative scent-related genes for association with the two QTL. Of 29 genes, only *ODO1* fulfilled all criteria. *ODO1* encodes a MYB-type transcription factor and antisense lines have reduced scent production [27]. *ODO1* maps to the QTL on chromosome VII and its expression correlates with scent production in wild accessions (Figure S5). Furthermore, in an interspecific F1, the expression from the *P. axillaris* allele was 17 times higher than from the *P. exserta* allele (Figure 3F). This indicates that the difference in expression is caused by a *cis*-acting polymorphism in *ODO1*. Initial studies suggest that this polymorphism is not situated within 1.2 kb upstream of the translation start site. Detailed promoter studies will be needed to identify its molecular nature.

Phylogenetic studies of the genus *Petunia* robustly place the small bee-pollinated species of the *P. integrifolia* group at the base of the tree, whereas *P. axillaris* and *P. exserta* are derived species [15]. The latter two species are very closely related and are not well resolved, although biogeographic information makes it perhaps more likely that *P. exserta* evolved from a *P. axillaris* ancestor. If this is the case, scent production first evolved in *P. axillaris* and was subsequently lost in *P. exserta*. A previous cross between *P. axillaris* S7 and *Petunia hybrida* identified QTL on chromosome VII, similar to this work [17]. Because all *P. hybrida* accessions are derived from a cross between *P. integrifolia* and *P. axillaris* [15], it is possible that acquisition and loss of scent involved the same genetic loci. More detailed mapping will resolve this interesting issue.

It has been previously established that visual and olfactory cues act synergistically on hawkmoths [11, 38, 41]. In our wind tunnel experiments, we observed that when scented plants were completely hidden, the moths responded more slowly. However, we observed increased antennal movements and proboscis unrolling when scent was present in the tunnel. It is therefore obvious that in such an experimental setup, both a visual and a scent cue are required to elicit a strong reaction by the moths.

We were also interested in determining how much hawkmoths use volatile emissions of plants to make choices at short distances. In our experiments moths clearly preferred plants that produced scent over scentless plants, even if these plants were positioned very close to each other. This resembles the position of sibling plants in a population. We therefore conclude that odor production is important for a moth's first choice within a plot and might be significant in determining the outcrossing efficiency for a plant. How much a wind tunnel setup resembles a natural population and how crucial the scent is for a plant's fitness will have to be tested in field experiments. The availability of introgression lines offers new opportunities to do so.

When we presented scented red flowers and nonscented white flowers to the hawkmoths, they appeared to be confronted with strongly conflicting cues. The fact that they could not make a choice between white flowers and scented flowers means that both traits are almost equally important for the attraction of the insect. That the volatiles have an additional function, namely to stimulate the attracted animal to feed on the flower, is manifested by the higher number (66%) of feeding events on the red, scented plants.

The advances in genomics technology now make it possible to link behavioral information to genetic and ecological knowledge [43]. We have identified two major genetic loci that specify the presence of scent in *P. axillaris* as compared to its absence in *P. exserta*. This rather simple genetic architecture should allow for a quick selection for scented plants in the rare case of hybridization. That hybridization occurs is well documented [22], but scented, red *Petunia* have not yet been reported. A complete molecular description of the genetic architecture of all the traits conditioning pollinator visitation will help in understanding how shifts in pollination syndromes occurred so frequently in many taxa.

Experimental Procedures

Plant Material

P. axillaris N and *P. axillaris* S7 were kindly supplied by R. Koes, Vrije Universiteit Amsterdam. *P. axillaris parodii* is referred to as P. parodii S7 by Wijsman [45] and Quattrocchio et al. [44]. *P. exserta* was a gift from R. J. Griesbach, Beltsville, MD. Other *P. axillaris* accessions were collected in Uruguay and Argentina (Figure S5).

Plants were grown in a greenhouse or in a growth chamber. Greenhouse plants were grown with additional lighting resulting in at least a 14 hr day (somewhat longer in summer) at 18–25°C in pots. Plants in the growth chamber were grown with a 15:9 light:dark cycle at 22°C during the day and 17°C during the night in pots or trays of 24 plants. Commercial soil (70% Klasman substrate, 15% Seramis clay granules, 15% quartz

sand) was fertilized once a week (Nitrogen-Phosphorous-Potassium and Iron).

Breeding of Introgression Lines

For the nonscented *P. axillaris* with a chromosome II introgression, we used a white, nonscented F2 *P. exserta* × *P. axillaris* S7 plant and backcrossed it to *P. axillaris* S7. The resulting F1 was selfed and scent measured for 24 F2 plants. After genotyping at least two markers per chromosome, we selected a plant that contained the smallest introgression from *P. exserta* and selfed it three more times. Each time 24 plants were measured with the PTR-MS and the scented plants with the smallest introgression (more markers were used on chromosome II) were selected. A selected plant was backcrossed once more and then selfed to yield the introgression lines WS and WNS (see Figure S3). Measuring scent at each generation indicated that a single locus was segregating. Line WS was heterozygous and line WNS homozygous *P. exserta* for markers cn9140, PAL1, FLS, PM134, and PM21. Both lines were homozygous *P. axillaris* for all 29 other markers tested.

Red, scented lines were bred by selecting the scented lines with the strongest color among the *P. exserta* S7 × *P. axillaris* S7 population and were backcrossed four times. At each generation, genotyping was performed to select for plants that had the smallest possible chromosome fragments from the *P. axillaris* parent. Siblings of the F2BC4 were used as introgression lines RS and RNS (see Figure S3). Line RS is heterozygous for *PAAS2a*, *Myb3*, *Myb58*, *FLS*, *PAL1*, *Myb14*, *cn9140*, and *IGS* on chromsome II and *PAL2b*, *MybB*, *ODO1*, and *COS13* on chromosome VII. Line RNS is heterozygous for *PAL2b*, *MybB*, *ODO1*, and *COS13* on chromosome VII. All other tested CAPS markers (34 in total) were homozygous for *P. exserta*.

The composition of the volatile production of red introgression lines was comparable to the *P. axillaris* parent, indicating that the loci on chromosome II and VII are necessary and sufficient to produce a *P. axillaris* scent bouquet. Although for the locus on chromosome II no quantitative difference was observed for plants heterozygous or homozygous for the *P. axillaris* allele, plants that were homozygous *P. axillaris* on chromosome VII produced somewhat more volatiles than heterozygous plants. The amounts produced by these introgression lines were less than 50% of the *P. axillaris* parent, indicating that other regions of the genome may be contributing to the full quantity of scent production. However, they do not have a sufficiently large effect to constitute a major QTL.

Insects

M. sexta pupae were obtained from the Laboratory of Animal Physiology (Philipps University, Marburg, Germany) and held under the following climatic conditions. Light was provided by six fluorescent tubes (Philips TDL, 36 W, >1 kHz) with a 16:8 light:dark cycle in an environmental cabinet programmed at 26°C and 65% relative humidity (RH). Pupae were sexed prior to emergence and placed in rearing cages (BugDorm-4180F, http://www.megaview.com.tw) containing a wet tissue. Naive adults used for the experiments were 4 to 6 days old, unmated, and unfed.

Wind Tunnel

The climatized wind tunnel (working area: 250 × 100 × 100 cm, see Figure 4A) is made of nonreflecting glass. A centrifugal ventilator moves the humidity- and temperature-controlled air (85% ± 1% RH, 26 ± 0.1°C) across the tunnel at 56 cm/s through active charcoal cartridges placed at either end of the working area. For our experiments, a laminar air flow was produced through a white nylon laminar flow screen (50 μm mesh) and two aluminum honey comb screens (6 cm thick, 1 cm mesh) covered with white mosquito netting (nylon, 1 mm mesh) placed upwind and a second white nylon laminar flow screen (50 μm mesh) at the downwind end of the wind tunnel. Overhead illumination was provided by high-frequency fluorescent lighting 186 cm above the tunnel floor (36 W, >1 kHz, with eight 120 cm long Philips TL-D tubes) running the length of the tunnel in two groups of four tubes 27 cm apart in a housing that was 120 cm wide (with fans to eliminate heat). Brown paper was placed on the top of the wind tunnel to decrease light intensity to 2–4 lux. The floor of the wind tunnel was covered with a medium density fiberboard (4 mm thick, light brown), and the white side walls had vertical light blue stripes 7 cm wide and 7 cm apart. Two plants similar in shape with the same number of flowers stood 40 cm from each other, 30 cm from the upwind end of the wind tunnel. Plant height was adjusted to place the flowers at 50 cm from the floor. Plant positions were systematically switched to avoid experimental bias.

Both insects and plants were tested at the beginning of the scotophase. Insects were placed in the darkened wind tunnel room 20 min prior to the experiments. Single quiet naive moths were placed on a 50 cm high platform at the downwind end of the tunnel. If the moths were not active (wing fanning) after 1 min, they were gently prodded by brushing the tip of their abdomen with a gloved finger. First choices for flowers, proboscis extension, and feeding were observed over 5 min. Differences between plants in terms of first choice and proboscis extension made by *M. sexta* were compared by using the exact binomial test in R.

Volatile Analysis

For proton transfer reaction mass spectroscopy (PTR-MS) analysis flowers of day 2 from anthesis were measured at sunset by cutting them and putting each in a glass vial. Fresh, reconstituted air (Carbagas, Bern, Switzerland) was supplied from a compressed air bottle at a rate of 50 l per hour to feed the inlet of the PTR-MS. A valve was constructed to prevent ambient air from entering the system and glass vials were screwed on the valve for volatile measurement with a PTR-MS (HS-PTR-QMS, Ionicon, Innsbruck, Austria) for 20 cycles per sample, corresponding to roughly 20 s of measurement. Raw data were transformed into concentrations (parts per billion [ppb]) with the Ionicon software PTR-MS control and PTR-MS viewer. To exclude day-to-day environmental variation, single measurements were normalized by dividing the concentration values by the average measurement value for the session (more than 100 plants were measured per session). Normalized values were used for calculating the average for each plant.

For GC, single flowers of day 2 from anthesis were enclosed at the beginning of the scotophase in an ~500 ml chamber made of polyethylene foil tied around the flower stem. Pure air was vented at 2 L/min through the chamber and 1 L/min was pulled for 120 min through 25 mg 80/100 mesh Porapak Super Q (Altech Assoc., Deerfield, Ill., USA) in a glass column previously conditioned under N2 at 200°C for 90 min. The remaining air in the chamber was evacuated through a hole so there was always surplus pressure in the collection chamber to avoid air entry via the enclosure on the plant stem. Flower effluent was desorbed from the adsorbent with dichloromethane (SupraSolv, Merck, Germany) and the 10 first μl collected. This represented over 95% of all volatiles trapped with the exception of benzaldehyde (60%).One μL of fragrance samples was analyzed by GC-FID (5300 chromatograph, Carlo Erba Instruments, Italy) mounted with a ZB-5 (Zebron, USA) apolar column (see below). Column conditions were 40°C for 5 min then increased at 3°C/min to 230°C and held for 5 min. Retention times of individual fragrance constituents present in *P. axillaris* [28] were compared to those of standards and confirmed by GC-MS (Trace GC-PolarsiQ, Finnigan, USA) on a ZB-5MS apolar column (30 m, 0.25 mm inner diameter, Zebron).

Measurement of Additional Flower Traits

Surface areas were measured by using front view photographs and analyzed with ImageJ (www.imagej.org). Nectar was weighed after extracting it from flower tubes by centrifugation. Flavonols and anthocyanin were extracted from petal tissue of 8 mm diameter with 1 ml of a water:methanol:acetate mix of 7:2:1. Absorption measurements are integrated values from a spectrophotometer of wavelengths 315 nm–378 nm for flavonols and 445 nm–595 nm for anthocyanins. Reflectance spectra were also performed and the results were in line with the absorption spectra.

DNA/RNA Manipulations

Plant DNA was isolated either by using a C TAB protocol as described [46] or with a plant GenElute DNA isolation kit (Sigma, Buchs, Switzerland). DNA was quantified with a Nanodrop ND-1000 (Thermo Fisher, Wilmington, Delaware, USA) and diluted to 100 ng/μl for CAPS and to 25–50 ng/μl for SSR amplification.

PCRs and cloning for CAPS markers was performed as described elsewhere [47]. Oligonucleotides (Microsynth, Balgach, Switzerland or Sigma, Buchs, Switzerland) and PCR conditions employed are described on our website (http://www.ips.unibe.ch/deve/caps/index.html). Mapping and QTL analysis were performed with QTX [48] for marker regression (free regression model without background control for other QTLs) and Q-gene (http://coding.plantpath.ksu.edu/qgene/download.html) programs. Interval mapping was performed with simple interval mapping function and significance thresholds were determined by resampling with 10,000 reiterations.

RNA employed in blotting experiments and Illumina sequencing was isolated from petal tissue 2 days after anthesis with Trizol according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA). RNA gel blots were performed as described [49]. Radioactive probes were prepared with a Megaprime labeling kit (GE Healthcare-Amersham)

according to the manufacturer's recommendations. Reverse transcripts were produced with M-MLV (Promega, Madison, USA) according to the manufacturer's recommendations.

Transcript Profiling

Total RNA was isolated from an F1 *P. exserta* x *P. axillaris* S7 and sent for cDNA production and sequencing to Fasteris (Plan-les-Quates, Switzerland). FastQ data were transformed to FastA sequences and then searched by making a local database by using BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The database was searched with short stretches of nucleotides (40–50 nts) containing polymorphisms for the two parent species. For each cDNA two query sequences were used, and the ratios of the perfect hits for each species were averaged (*EOBII* has only one polymorphism). The number of hits (*P. axillaris*:*P. exserta*) were 1518:102, 367:299, 35:54, 108:98, and 2069:1455 for *ODO1*, *EOBII*, *PAL1*, polyubiquitin (GenBank acc DC242775), and GAPDH (GenBank acc FN016442) alleles, respectively.

Supplemental Information

Supplemental Information includes Supplemental Discussion and five figures and can be found with this article online at doi:10.1016/j.cub.2011.03.059.

Acknowledgments

We thank R. Koes, F. Quattrocchio, R.J. Griesbach, and B. Schlumpberger for plant materials; A. van Moerkercke and R. Schuurink for making the sequence of the ODO1 promoter available; and M. Held for advice on PTR-MS. We thank L. Freitas and A. Segatto and all the members of the Kuhlemeier group for helpful discussions and C. Ball, R. Alder, and N. Signer for taking care of the plants. We also thank A. Vallat, Analytical Service of the Faculty of Science, Institute of Chemistry, University of Neuchatel for the GC-MS analyses of flower fragrance. This work was financially supported by the National Centre for Competence in Research "Plant Survival" and the Universities of Bern and Neuchâtel.

Received: February 4, 2011
Revised: March 18, 2011
Accepted: March 22, 2011
Published online: April 14, 2011

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