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Report

# Tight Genetic Linkage of Prezygotic Barrier Loci Creates a Multifunctional Speciation Island in *Petunia*

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## Summary

**Most flowering plants depend on animal vectors for pollination and seed dispersal. Differential pollinator preferences lead to premating isolation and thus reduced gene flow between interbreeding plant populations [1–4]. Sets of floral traits, adapted to attract specific pollinator guilds, are called pollination syndromes [5]. Shifts in pollination syndromes have occurred surprisingly frequently [6], considering that they must involve coordinated changes in multiple genes affecting multiple floral traits. Although the identification of individual genes specifying single pollination syndrome traits is in progress in many species, little is known about the genetic architecture of coadapted pollination syndrome traits and how they are embedded within the genome [7]. Here we describe the tight genetic linkage of loci specifying five major pollination syndrome traits in the genus *Petunia*: visible color, UV absorption, floral scent production, pistil length, and stamen length. Comparison with other Solanaceae indicates that, in *P. exserta* and *P. axillaris*, loci specifying these floral traits have specifically become clustered into a multifunctional "speciation island" [8, 9]. Such an arrangement promotes linkage disequilibrium and avoids the dissolution of pollination syndromes by recombination. We suggest that tight genetic linkage provides a mechanism for rapid switches between distinct pollination syndromes in response to changes in pollinator availabilities.**

## Results and Discussion

*Petunia* is a classical genetic model system and includes species that attract different pollinators, making it ideally suited to study the genetics underlying pollinator-driven speciation [10–12]. *Petunia exserta* has a hummingbird pollination syndrome with red, UV-reflecting, nonscented petals and exserted reproductive organs, whereas the hawkmoth-pollinated *P. axillaris* has white, UV-absorbing, volatile-producing petals and nonexserted reproductive organs [13, 14] (Figure 1). The two sister species have been found growing in sympatry with occasional hybridization. It has been suggested that *P. exserta* is derived from *P. axillaris*, based on molecular and biogeographic data [13, 15], and thus may represent a case of sympatric speciation. Previously, we identified two major quantitative trait loci (QTL) for floral scent production in the $F_2$ progeny of an interspecific cross between *P. axillaris* and *P. exserta.* These two QTL, on chromosomes II and VII, are sufficient for floral scent production when introgressed into the *P. exserta* genetic background. The locus on chromosome VII was identified as the MYB transcription factor *ODORANT1* [16]. For fine-mapping purposes, we introgressed both QTL into the *P. exserta* genetic background and selected a line, named "introgression line 1" (IL1), that was heterozygous for the chromosome II QTL and homozygous for *P. axillaris* on chromosome VII. As expected, the selfed progeny of this line segregated 3:1 for the presence or absence of floral scent.

Unexpectedly, IL1 segregated not only for scent but also for visible color, UV absorption, and male and female reproductive organ morphology. To determine the genetic distance between the loci involved, we analyzed 504 progeny plants, of which 503 segregated into three distinct phenotypic classes. The first class (120 plants, homozygous for *P. axillaris* at the chromosome II introgression) had white, UV-absorbing, and scented flowers with short pistil and stamens, similar to the *P. axillaris* parent. The second class (124 plants, homozygous for *P. exserta* at the chromosome II introgression) resembled the *P. exserta* phenotype with red, UV-reflecting, and unscented flowers with elongated pistil and stamens. The third class (259 plants, heterozygous at the chromosome II introgression) was similar to the IL1 parent (Figure 1). The three phenotypic classes occurred in a ratio of 120:259:124 (1:2:1, $\chi^2 = 0.51$; $p = 0.48$), consistent with a single Mendelian locus with the *P. axillaris* allele being dominant for UV absorbance, visible color, and scent production and the *P. exserta* allele being partially dominant for pistil and stamen elongation (Figure 1B).

In order to determine whether the locus represents a "master" gene with pleiotropic effects or consists of multiple genes, we searched for rare recombinants in the segregating population described above as well as related crosses. A single plant was found in the described population that resembled *P. exserta* for floral color, UV reflectance, and pistil and stamen morphology but produced floral scent (Figure 2: IL1-1). Genotyping of this plant confirmed a breakpoint between the markers FLS and MYB14. In addition, it located the chromosome II regulator for floral scent production between the EOBII and MYB14 markers (Figure 2: IL1-1). One recombination event in 1,008 chromosomes defines a genetic distance of 0.1 centimorgans (cM) between the floral scent production locus and one or more loci specifying the other three floral traits. We were able to separate determinants of scent and color production as well as reproductive organ morphology in additional independently generated introgression lines (for details, see Figure S2 available online). IL2 resembles IL1, except that it is *P. exserta* homozygous for chromosome VII. Therefore, it segregates for visible color, UV absorption, and male and female reproductive organ morphology, but not for floral scent. Further backcrossing led to IL2-1, segregating only for color and UV absorption but no longer for pistil and stamen lengths (Figure 2: IL2-1). Thus, major regulator(s) for visible and UV color production were separated from regulator(s) shaping reproductive organ morphology. A recombination breakpoint was found between the markers FLS and MYB14 (Figure 2: IL2-1). In a further cross (IL3), the chromosome II locus was introgressed into the genetic background of *P. axillaris*. IL3 segregated for all five pollination syndrome traits, albeit not for the entire parental

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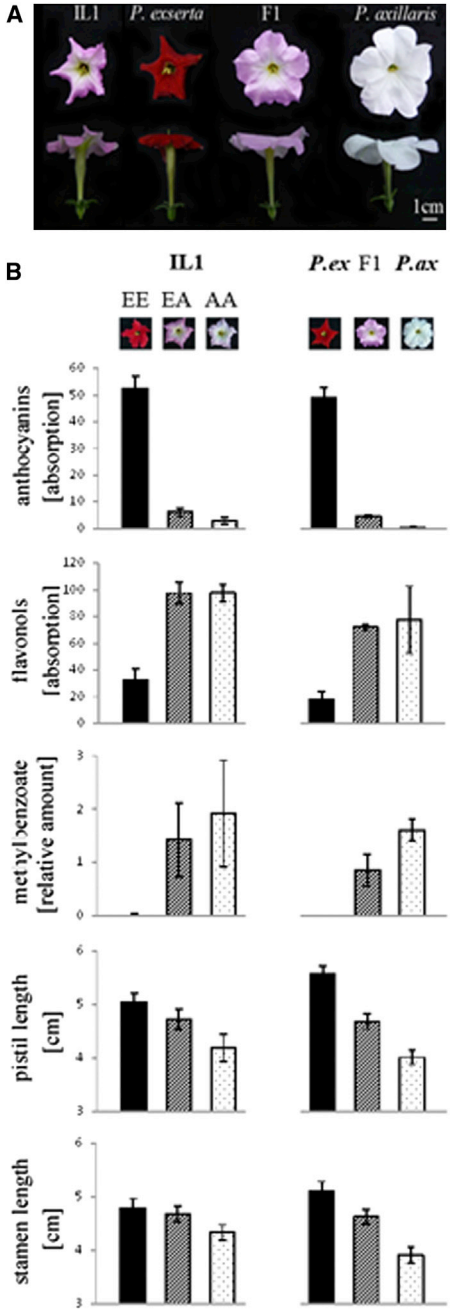


Figure 1. A Single Locus on Chromosome II Segregates for Five Distinct Pollination Syndrome Traits

(A) Top and side views of IL1, the interspecific $F_1$ hybrid, and the two parental species *P. exserta* and *P. axillaris*.

(B) The left panel shows the segregating progeny of IL1, and the right panel shows the two parents and their $F_1$ hybrid. Anthocyanin and flavonol content, relative methylbenzoate production, and pistil and stamen length measurements are shown as mean values of at least three measurements from five different plants. Error bars represent SDs. EE, homozygous for *P. exserta* at the chromosome II locus; EA, heterozygous; AA, homozygous for *P. axillaris* at the chromosome II locus. All five floral traits cosegregate in 503 progeny plants of IL1. Measurement details are given in Experimental Procedures.

phenotypic variance, presumably because additional loci are needed in this genetic background for anthocyanin production and the elongation of pistil and stamens. IL3-1, a further backcrossed progeny, no longer segregated for color and reproductive organ morphology, but did segregate for scent production (Figure 2: IL3; IL3-1). Taken together, the recombination breakpoints define at least three independent loci (Figure 2C).

Pleiotropic master regulators, tight genetic linkage, and reduced recombination have been postulated to be required for coordinated evolution at key loci during speciation with gene flow [17–19]. The recombinants we observe exclude a pleiotropic master regulator as well as suppression of recombination by a single inversion. Our data favor the existence of a gene cluster comprising very tightly linked, yet independent, regulators shaping the major pollination syndrome traits of visible and UV color production, scent, and reproductive organ morphology. Visible color and UV absorption, as well as male and female organ morphology, could not be separated by recombination. In the case of the visible color and UV traits, these may be under the control of separate genes or regulated by a common regulator that activates flavonol and represses anthocyanin biosynthesis. The opposite effects of this locus might also be the result of competition for a common substrate, given that both flavonols and anthocyanins derive from the amino acid phenylalanine. Because elongation of male and female organs is not fully synchronous, separate control of these traits seems more likely.

To determine the history of the cluster, we blasted the *Petunia* sequences to the two currently available, fully assembled Solanaceae genome sequences of tomato (*Solanum lycopersicum*) and potato (*S. tuberosum*) [20, 21]. Homologs of genes containing the ten clustered *Petunia* markers are distributed over six chromosomes in tomato and five chromosomes in potato (Figure 3; Figure S3). Thus, the cluster is specific for *Petunia.* Homologs of four markers (PAL1, pm134, MYB14, and DA1-l) are widely spaced on chromosomes V of tomato and potato with combined lengths of 6.04 Mbp and 7.89 Mbp, respectively (Figure 3; Figure S3 and Tables S1 and S2). On tomato chromosome V, the combined distance between the markers PM134, PAL1, and DA1 is 2.18 Mbp, equivalent to 20 cM in this region of the tomato genome [22]. In the absence of a fully assembled *Petunia* genome sequence, it is difficult to determine the physical size of the cluster. The combined size of the *P. axillaris* genome scaffolds containing the ten clustered markers is 0.64 Mbp. This is only a minimal estimate based on ten markers, and the actual physical size is likely to be much larger. Indeed, fluorescence in situ hybridization data from *P. hybrida* suggest that the FLS marker maps to a pericentric region with low recombination [23].Thus, we favor the hypothesis that the linkage of traits in *Petunia* is primarily due to the suppression of recombination associated with heterochromatic regions.

Changes in pollination syndrome traits can alter pollinator attraction leading to reproductive isolation [2–4, 16]. We identified tight genetic linkage between five prezygotic barrier loci, creating a multifunctional speciation island. Linkage of coadaptive loci has been described in other species, often involving genomic inversions or rearrangements [9, 19, 24–27]. Such linkage facilitates the cosegregation of coadaptive variation and limits the production of unfit recombinant forms. Two hypotheses have been put forward to explain the evolution of linkage [28–31]. The first is that loci were at first unlinked and then brought close together after the polymorphism arose; this might involve chromosomal rearrangements or selection for mutations in genes controlling the rate of recombination. The second hypothesis is that loci

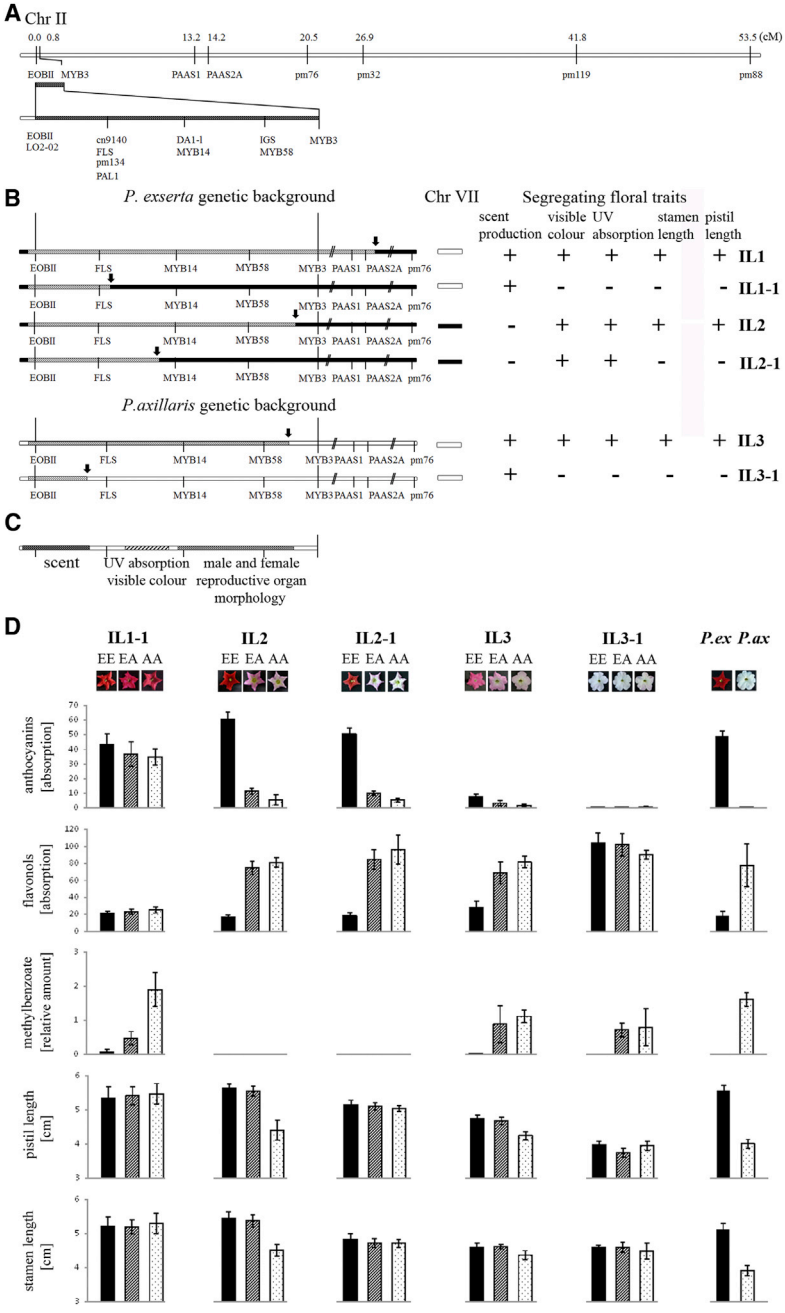


Figure 2. The Linkage of Floral Traits Can Be Broken Up by Recombination

(A) Marker distances in centimorgans (cM) were obtained from an $F_2$ mapping population of an interspecific cross between *P. axillaris* and *P. exserta* (see also Figure S1). Markers for chromosome II are shown here. For all introgression lines, a magnified view is shown between the markers EOBII and MYB3. Black bars refer to genomic regions homozygous for *P. exserta*, and white bars refer to genomic regions homozygous for *P. axillaris*. For better visualization, marker intervals are not to scale.

(B) All introgression lines segregate for the chromosome II locus. IL1 and its progeny line IL1-1 are in the *P. exserta* background but homozygous for *P. axillaris* at the scent QTL on chromosome VII. IL2 and its progeny IL2-1 are homozygous for *P. exserta* at chromosome VII and therefore do not segregate for scent. IL3 and its progeny IL3-1 are in the *P. axillaris* background. Plus (+) indicates segregating; minus (−) indicates nonsegregating. Introgressed regions are indicated hatched; recombination break points are highlighted by arrows.

(C) The location of major regulators for scent, UV absorption, visible color production, and reproductive organ morphology.

(D) Anthocyanin and flavonol content, relative methylbenzoate production, and pistil and stamen length measurements are shown as mean values of at least three measurements from five different plants. Error bars represent SDs. EE, homozygous for *P. exserta* at the chromosome II locus; EA, heterozygous; AA, homozygous for *P. axillaris* at the chromosome II locus.

were tightly linked from the beginning and that mutations had a selective advantage due to being linked (selective sieve). In *Petunia*, substantial rearrangements have taken place since the divergence from tomato and potato [32]. The time of divergence between tomato and potato is estimated to be 7.3 million years ago (mya), whereas their common ancestor split from the *Petunia* lineage approximately 30 mya [33]. Given that species in the genus *Solanum* are generally pollinated by bees [34], divergence of pollination syndromes has clearly not been a major factor in the massive species-level radiation of *Solanum* [35]. At present, it is not known to what extent the adaptive mutations described here arose before or after the genomic rearrangements. In order to resolve this issue, we have embarked on the identification of the genes involved as well as their genomic architecture in *Petunia* and related taxa.

*P. exserta* is an endangered species growing in a restricted area in the Serra do Sudeste region in southeastern Brazil, where it occasionally hybridizes with the much more abundant *P. axillaris* [13]. We hypothesize that strong selection on the genetically clustered floral traits generally keeps the two species separated and maintains *P. exserta*'s distinctness as a species. On the other hand, close linkage of genes encoding the complex of traits involved could allow the pollination syndrome to switch through hybridization in just a few generations. This will greatly aid adaptation to rapid environmental changes, such as shifts in the availability of pollinators.

### Experimental Procedures

Introgression lines were created through repeated backcrosses and selfings starting from individual $F_2$ plants derived from an interspecific cross between a *P. axillaris* N and *P. exserta*. *P. exserta* is naturally self-compatible, whereas *P. axillaris* is mostly self-incompatible in nature. *P. axillaris* N may represent a rare self-compatible accession or may have lost incompatibility during greenhouse propagation. An overview of the breeding schemes is given in Figure S2. Complete marker and mapping information can be found in Bossolini et al. [32], Figure S1, and http://www.botany.unibe.ch/deve/caps/index.html.

For phenotypic measurements, at least three flowers per progeny plant were sampled 1 day after flower opening, 1 hr before the end of the day cycle in the growth room. All progeny plants were sown simultaneously and phenotyped after the first three flowers had opened. We measured all five phenotypic traits in each flower starting with male and female reproductive organ morphology, followed by scent, then visible and UV color measurements.

To determine reproductive organ morphology, we cut the flowers open along their dorsal-ventral axis and photographed them in order to extract pistil length, stamen length, and D1 tube length data. Each image was processed using ImageJ [36]. Anthocyanins and UV-absorbing flavonols were

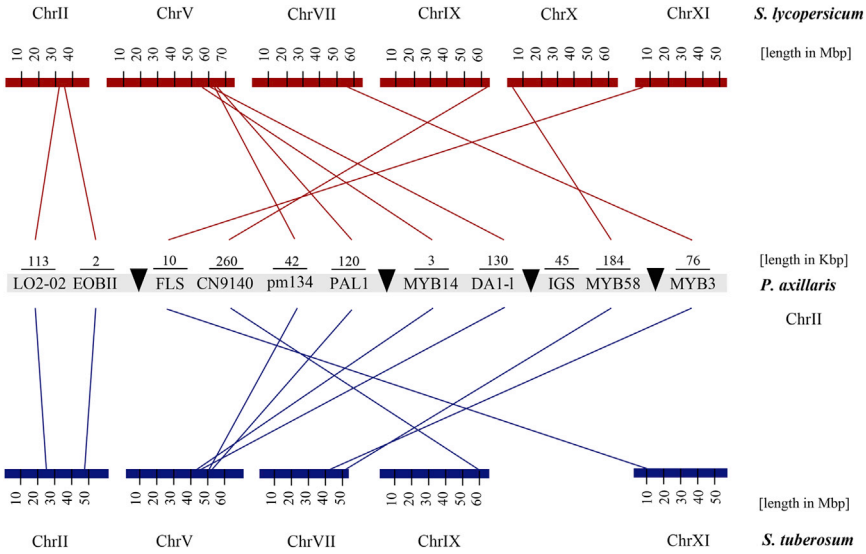


Figure 3. Clustering Is *Petunia* Specific

Marker-derived gene sequences within the cluster were blasted against predicted cDNA sequences for *S. lycopersicum* and *S. tuberosum*. The clustered *Petunia* markers map to six different chromosomes in tomato and five different chromosomes in potato. Note that 9 out of 10 individual markers map to homologous tomato and potato chromosomes. Distances are shown in Mbp for tomato and potato and kbp for *P. axillaris* scaffolds. Recombination breakpoints found in different introgression lines are indicated by triangles; marker orders in between are arbitrary. For details, see also Figure S3 and Tables S1 and S2.

measured by scanning spectrophotometry. Volatiles were determined by proton transfer reaction-mass spectrometry (PTR-MS).

Molecular marker sequences that mapped to the cluster were blasted against the *P. axillaris* draft genome scaffolds (v0.1.1). For each scaffold, the corresponding gene sequences were predicted with RNA-seq data using cufflinks (v2.0.2). The gene sequence was used for blast analysis against the predicted cDNA sequences for *S. lycopersicum* and *S. tuberosum*.

Details of the Experimental Procedures are given in the Supplemental Experimental Procedures.

## Supplemental Information

Supplemental Information includes three figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.03.069.

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