

The genetics of reproductive organ morphology in two *Petunia* species with contrasting pollination syndromes

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Abstract

Main conclusion Switches between pollination syndromes have happened frequently during angiosperm evolution. Using QTL mapping and reciprocal introgressions, we show that changes in reproductive organ morphology have a simple genetic basis.

In animal-pollinated plants, flowers have evolved to optimize pollination efficiency by different pollinator guilds and hence reproductive success. The two *Petunia* species, *P. axillaris* and *P. exserta*, display pollination syndromes adapted to moth or hummingbird pollination. For the floral traits color and scent, genetic loci of large phenotypic effect have been well documented. However, such large-effect loci may be typical for shifts in simple biochemical traits, whereas the evolution of morphological traits may involve multiple mutations of small phenotypic effect. Here, we performed a quantitative trait locus (QTL) analysis of floral morphology, followed by an in-depth study of pistil and stamen morphology and the introgression of individual QTL into reciprocal parental backgrounds. Two QTLs, on chromosomes II and V, are sufficient to explain the interspecific difference in pistil and stamen length. Since most of the difference in organ length is caused by differences in cell number, genes underlying these QTLs are likely to be involved in cell cycle regulation.

Interestingly, conservation of the locus on chromosome II in a different *P. axillaris* subspecies suggests that the evolution of organ elongation was initiated on chromosome II in adaptation to different pollinators. We recently showed that QTLs for pistil and stamen length on chromosome II are tightly linked to QTLs for petal color and volatile emission. Linkage of multiple traits will enable major phenotypic change within a few generations in hybridizing populations. Thus, the genomic architecture of pollination syndromes in *Petunia* allows for rapid responses to changing pollinator availability.

Keywords Mating system evolution · Near-isogenic lines · QTL · *Petunia* · Reproductive organ morphology · Speciation

Abbreviations

BLAST	Basic local alignment search tool
CAPS	Cleaved amplified polymorphic sequence
IL	Introgression line
LOD	Logarithm of the odds
PVE	Phenotypic variation explained
QTL	Quantitative trait locus

Introduction

Most flowering plants are hermaphroditic, with flowers that contain both male and female sexual organs. While some species are predominantly self-fertilizing, others avoid inbreeding by outcrossing or mixed-mating strategies. Each mating strategy is characterized by a set of features that maximizes mating success. In animal-pollinated plants,

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parallel evolution of adaptations to pollinators is found. This generates patterns of common characters, called ‘pollination syndromes’, to which flowers of quite different evolutionary origins may converge. The resulting divergence in floral phenotypes can lead to pollinator-mediated reproductive isolation which is a prerequisite for speciation (Hodges and Arnold 1994; Kay and Schemske 2003; Fenster et al. 2004; Gegear and Burns 2007; Thomson and Wilson 2008). Thus, the genes involved in switches between pollination syndromes are potential speciation genes.

Pollination syndrome traits have been divided into: (i) attraction traits such as color, fragrance, flower shape and size, (ii) rewarding traits such as nectar volume, composition and concentration and (iii) efficiency traits, defined as characters influencing pollen removal and deposition, in particular reproductive organ morphology (Bradshaw et al. 1995). Changes in each of these traits are likely to require modifications in at least one gene. In the case of petal color, by far the best studied trait, single loci or single genes affect pollinator behavior (Bradshaw and Schemske 2003; Hoballah et al. 2007; Streisfeld and Rausher 2009; Hopkins and Rausher 2011; Yuan et al. 2013). Similarly, polymorphisms in two loci controlling scent production affect pollinator change (Klahre et al. 2011). Thus, modifications of petal color and scent production involve a few genes of medium to major effect. Color and scent both involve comparatively simple pathways of secondary metabolism (Sheehan et al. 2013). Mutations in such pathways may cause little pleiotropy and thus permit mutations of large effect (Rockman 2012; Martin and Orgogozo 2013).

Many QTL studies have been undertaken to identify the genetic loci for reproductive organ morphology in genera comprising species with distinct pollination syndromes. In contrast to modifications of petal color and scent production, changes in morphological characters may be more likely to involve multiple smaller effect loci (Brothers et al. 2013; Nakazato et al. 2013; Wessinger et al. 2014). However, none of the identified QTLs has been defined further nor have the underlying genes been identified. The genetic basis of reproductive organ morphology is most extensively studied with respect to the ‘selfing syndrome’, where a set of morphological changes is associated with the switch from outcrossing to self-pollination (Barrett 2010; Sicard and Lenhard 2011; Sicard et al. 2011). In tomato, a series of QTL mapping experiments revealed five tightly linked loci controlling style and stamen length underlying a complex QTL, named *stigma exertion 2.1* (Bernacchi and Tanksley 1997; Fulton et al. 1997; Chen and Tanksley 2004). Fine mapping of the *se.2.1* locus identified the *LO2* gene and allelic variation at the 5′ regulatory region was found to modulate style length and, thus, stigma exertion. Thus, a single QTL breaks up in multiple individual genes that are closely linked.

A key question to be answered concerns the nature of the genetic changes underlying shifts in pollination syndromes. Do genes of large effect as found in QTL studies predominate? Or are they the exception and are numerous small mutations the norm? We have set out to identify the major genes involved in shifts between pollination syndromes in the genus *Petunia*. Important for our goal, *Petunia* also is one of the classical genetic model systems with a wealth of sophisticated genetic, genomic and biochemical tools (Koes et al. 1995; Souer et al. 1995; Vandebussche et al. 2008; Gerats and Strommer 2009; Kanazawa et al. 2011). High-quality draft genomes of two wild species, *P. axillaris* and *P. inflata*, have been completed (*Petunia* Genome Consortium, data not shown), and the combination of interesting biology with powerful molecular tools makes *Petunia* an attractive study system. We have used this system to identify loci involved in petal color and floral scent production and shown that changes in each of these traits can change pollinator behavior (Hoballah et al. 2007; Klahre et al. 2011; Dell’Olivo and Kuhlemeier 2013). Recently, we discovered that a scent locus on chromosome II colocalized with QTLs for visible color, UV absorption, as well as style and stamen length within 0.1 centiMorgan (cM) (Hermann et al. 2013). Rare recombinants within this region showed that these traits are encoded by separate genetic loci that segregate as a unit. This tight genetic linkage was not found in the genomes of the two related *Solanaceae*, tomato and potato, and may have evolved in response to pollinators. Also in *Penstemon*, colocalization of major loci shaping floral traits has been found (Wessinger et al. 2014). To understand the origin as well as the evolutionary and ecological significance of this complex locus, it is necessary to identify the causative mutations underlying the phenotypic differences in each trait.

The objective of this study is to unravel the genetic basis of different reproductive organ morphologies in two *Petunia* species with contrasting pollination syndromes. We used a QTL approach to identify the underlying QTLs in combination with a candidate gene approach. We introgressed individual loci into reciprocal parental backgrounds to study their effect sizes and investigated their epistatic interactions in multiple introgressions. In addition, we performed a developmental analysis of differential organ growth to study the cellular basis of the different organ sizes. Such studies are an important step towards the dissection of pollination syndrome to the level of individual genes and the causative mutations.

Materials and methods

Study system and experimental crosses

Petunia exserta is found in a small number of shallow caves in sandstone towers in the Serra do Sudeste region in

southeastern Brazil (Stehmann 1987; Lorenz-Lemke et al. 2006; Segatto et al. 2014). It has a bird pollination syndrome, typified by red flowers with unscented petals and reflexed lobes, strongly exerted stamens and stigma, and the production of copious dilute nectar. *Petunia exserta* is genetically self-compatible but the specific reproductive organ morphology may hinder self-fertilization. *Petunia axillaris* grows in open habitats and has a broad distribution area ranging from the Andean slopes in Argentina to Uruguay and southern Brazil. It displays a typical hawkmoth pollination syndrome with white, rounded corolla lobes, a non-exserted stigma, and stamens of different lengths. The flowers emit a strong fragrance at dusk and produce considerable amounts of nectar. The two sister species are cross-compatible in the laboratory and have been found growing in allopatry and sympatry. In sympatry, *P. axillaris* grows only outside the shallow caves where *P. exserta* occurs. Hybridization and gene flow are present, presumably due to hummingbird visits to both species, while two genetic clusters corresponding to the two species are maintained (Segatto et al. 2014). The natural hybrids have been exclusively found inside the *P. exserta* habitat (Segatto et al. 2014).

Petunia a. axillaris N and *P. a. parodii* S7 were kindly supplied by R. Koes, Vrije Universiteit Amsterdam, NL. *Petunia exserta* was a gift from R.J. Griesbach, Beltsville, MD, USA. These lines were maintained by selfing and grown as described in (Klahre et al. 2011). While inbred lines are valuable for QTL mapping, it has to be acknowledged that they may complicate matters if there is segregation distortion that results from differential loss of deleterious mutations. Wild *P. a. axillaris* and *P. exserta* were sampled in Guaritas, Torrhinas and Pedra da Cruz, Rio Grande do Sul, Brazil and José Ignacio, Uruguay in November 2008. The geographic coordinates were obtained via the global positioning system (GPS) (Table S1). The F₂ mapping population used for the QTL analysis ($n = 203$) was generated from a single F₁ progeny of a cross between *P. a. axillaris* N (as female) and *P. exserta* (as male) (Klahre et al. 2011). Crossing details of single and multiple introgressions of the identified QTLs into both parental backgrounds are given in Fig. S1. In each generation, plants were selected according to their genotype and phenotype. The introgression of the QTL on chromosome II from *P. axillaris* N into *P. a. parodii* S7 started from a F₂ plant described in Venail et al. (2010). The introgression of the QTL on chromosome II from *P. exserta* into *P. a. parodii* S7 is described in the supplemental material of Klahre et al. (2011).

Phenotypic measurements of reproductive organs and corolla traits

Measurements of pistil length, dorsal, lateral and ventral stamen length, stigma surface area, ovary length, proximal

(D1) and distal (D2) tube length and limb size (D3) have been conducted 2 days post-anthesis in the parental species, the F₁ hybrid plants and the F₂ plants; all introgression lines as well as the wild populations. A minimum of three flowers per plant were sampled and each image was processed using image J (Abràmoff et al. 2004). Phenotypic means were calculated based on raw measurements (Table 1; Fig. S2). Fully open flowers (2 days after anthesis and after anther dehiscence) were photographed from side view for D2 tube length and D3 limb length measurements (Stuurman et al. 2004). Then, flowers were cut open along their dorsal–ventral axis and photographed to extract pistil length, stamen length and D1 tube length data. Subsequently, stigmas were cut off and photographed from top view. The differences in stamen lengths were calculated from the obtained data. A graphical description of the morphological measurements can be found in Fig. S2. Except for the phenotyping of natural populations, all plants were grown in the greenhouse in Bern.

Cell size measurements and developmental series

Since organ length measurements are destructive, it was not possible to measure growth of individual pistils over time. Therefore, we first measured pistil length and bud length at several time points in at least three different plants of *P. exserta* and *P. a. axillaris* N. The high correlation between pistil and bud length in both species ($R^2 = 0.995$ and $R^2 = 0.997$; data not shown) allowed us to stage pistils based on bud growth over time. For cell size measurements, three fully opened flowers of *P. exserta* and *P. a. axillaris* N were harvested and dissected to extract pistils. The pistils were mPS-PI stained following the protocol of Truernit et al. (2008). Each ovary was cut off below the point of union with the style and each stigma was cut off above the point of union with the style before imaging. Each style was kept in Hoyer's solution between a cover glass and a microscope slide and recorded as sequential TIF files using the Zeiss Axioskop 2 equipped with an Axiocam camera. The sequential images of each style were then merged into a single image using Photoshop software (CS4, Adobe Systems Inc.) and the length of each cell in three different cell files over the full length of each style was measured using ImageJ (Abràmoff et al. 2004). Stigma sizes were imaged using a scanning electronic microscopy and quantified using ImageJ.

Linkage mapping, QTL analysis and candidate genes

Mapping of previously developed simple sequence repeat (Bossolini et al. 2011) and (derived-) cleaved amplified polymorphic sequence (dCAPS, CAPS) (Klahre et al. 2011) markers resulted in marker intervals of 5–10 cM

Table 1 Floral traits measured in *P. exserta*, *P. a. axillaris* N and the F₁ hybrid

Trait	Abbreviation	<i>P. exserta</i> mean ± SD (cm)	<i>P. a. axillaris</i> N mean ± SD (cm)	F ₁ hybrid mean ± SD (cm)
Pistil length	pl	5.33 ± 0.26	3.99 ± 0.24	4.69 ± 0.15
Ventral stamen length	vsl	4.82 ± 0.15	3.40 ± 0.13	4.17 ± 0.15
Lateral stamen length	lsl	5.00 ± 0.17	3.65 ± 0.16	4.48 ± 0.12
Dorsal stamen length	dsl	5.12 ± 0.17	3.91 ± 0.15	4.63 ± 0.14
Difference dorsal and ventral stamen length	ddvsl	0.29 ± 0.09	0.51 ± 0.09	0.46 ± 0.10
Difference dorsal and lateral stamen length	ddlsl	0.12 ± 0.05	0.26 ± 0.08	0.15 ± 0.05
Proximal tube length	D1	2.05 ± 0.22	2.19 ± 0.17	2.36 ± 0.11
Distal tube length	D2	2.45 ± 0.15	2.08 ± 0.17	2.36 ± 0.16
Limb size	D3	2.02 ± 0.13	2.26 ± 0.22	2.40 ± 0.28
Stigma area (cm ³)	sa	0.015 ± 0.004	0.047 ± 0.005	0.030 ± 0.003
Distance dorsal stamen–stigma	ddss	0.20 ± 0.08	0.08 ± 0.05	0.07 ± 0.03

(Fig. S3). Linkage analysis was performed using QTX; kosambi mapping function (Manly et al. 2001). The seven linkage groups gave a total length of 449 cM. Simple interval mapping was performed for each trait using Qgene 4.2.3. (Joehanes and Nelson 2008), using phenotypic mean values of the F₂ progeny and ILs. Permutation analysis (1,000 iterations) was done for each linkage group to obtain LOD score significance thresholds.

Illumina-generated cDNA sequences of *P. exserta* and *P. a. axillaris* N (Hermann et al. 2013) and *P. a. axillaris* N-expressed sequence tags were used for local BLAST (blast.ncbi.nlm.nih.gov) of the style 2.1 sequence (GI:162280538) from tomato. CAPS markers were designed for three homologs (LO2-01; LO2-02; LO2-03) and mapped in the F₂ *P. a. axillaris* N × *P. exserta*. For marker details and mapping information, see <http://www.ips.unibe.ch/deve/caps/markers.html> and Fig. S3.

MYB14 expression

We studied the expression of Myb 14 because the marker developed in this gene was the only one associated with the identified QTL on chromosome II. Myb14 transcript levels were first assessed by semi-quantitative PCR. Five microliters of first strand cDNA (10 × dilution) were used as template for the subsequent PCR amplification with 30 cycles. For PCR and primer details, see http://www.botany.unibe.ch/deve/caps/myb14_caps.html. Actin was used as internal control with 27 cycles of amplification. At least three style samples per developmental stage of each species were investigated. Eight developmental stages were defined to depict uniform periods along the entire developmental series for pistils (Fig. 1b). Second, Illumina sequencing (HighSeq 2000) of *P. a. axillaris* N and *P. exserta* transcriptomes from stylar tissue of mixed

developmental stages was done by MacroGen Inc. and sequence assemblies by Data2Bio™. Three biological replicates were sequenced and resulted in an average of 18 million reads per sample. The reads were normalized according to the total amount of reads per sample, and were aligned using bowtie2 software.

Statistical analysis

All data were analyzed for normality using the Shapiro–Wilk test. As a normal distribution was violated in all cases, we conducted non-parametric tests (Kruskal–Wallis) to compare different categories (=genotypes). In case of a significant result, post hoc analyses (Mann–Whitney *U*) were performed to calculate the differences between categories. For all statistical analyses, we used SPSS 22.0 for Windows (SPSS Inc.).

Results

The exsertion of *P. exserta*'s stigma is caused by increased cell division in the style

Petunia exserta and *Petunia axillaris* flowers differ greatly in flower morphology. *P. exserta* has nearly radially symmetric flowers with five pointed petals that are reflexed. The circular stamens are all approximately the same length, are strongly exserted and the long style extends the stigma beyond them (Stehmann et al. 2009). *P. a. axillaris*, in contrast, has a non-reflexed, weakly bilateral flower with a non-exserted stigma and stamens that form three distinct layers with 0.3–0.5 cm difference in length (a dorsal and a lateral stamen pair and a single ventral stamen, Table 1; Fig. 2). The pistil length was 1.3 cm longer in *P. exserta*

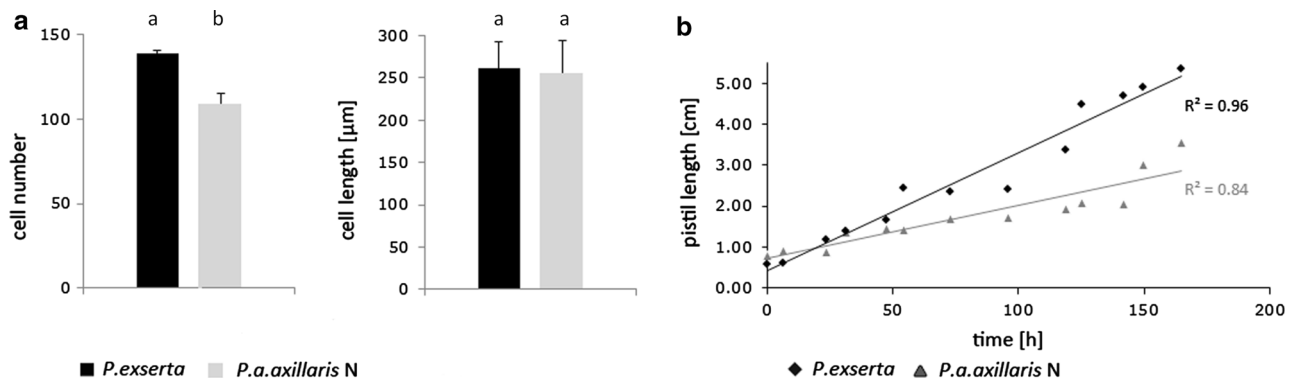


Fig. 1 Growth and cell size measurements of *P. exserta* and *P. a. axillaris N* pistils. **a** The average cell length in *P. a. axillaris N* and *P. exserta* styles is not significantly different, but *P. exserta* styles have significantly more cells than *P. a. axillaris N* styles. Error bars show standard deviations, different letters above bars

statistically different means ($P = 0.05$) as determined by the Mann–Whitney *U* test. **b** *P. exserta* pistils grow faster, but not longer than *P. a. axillaris N* pistils. The time scale was calculated from the correlation of pistil and bud growth (see “Materials and methods”). Values are means of three pistils per time point

than in *P. a. axillaris N* and the F1 hybrid showed an intermediate pistil length. Comparable results were found for stamen lengths with the longest, dorsal stamen pair being 1.2 cm longer in *P. exserta* than in *P. a. axillaris N*. Only a small difference of 0.2 cm was found for proximal (D1) plus distal tube length (D2). The combination of a negligible difference in length of the floral tube between the species with increased lengths of pistil and stamens results in the exertion of male and female reproductive organs in *P. exserta*. Stigma surface area was three times smaller in *P. exserta* than in *P. a. axillaris N* and scanning electron microscopy showed that the stigma of *P. exserta* had longer papillae than *P. a. axillaris N* (data not shown). Reproductive organ morphology is pollination independent in *Petunia* (Fig. S4).

P. a. axillaris N (Fig. 1a). Thus, the elongated *P. exserta* styles have more cells and this explains at least 0.8 cm of the interspecific pistil length difference of 1.3 cm.

How does the difference in cell numbers come about? Do styler cells proliferate faster in *P. exserta* than in *P. a. axillaris N*, or for a longer period? To address this question, we followed pistil growth in both species. A higher growth rate for floral buds and pistils was found in *P. exserta*, but duration of growth was similar, indicating faster growth of these organs throughout pistil development (Fig. 1b). Therefore, gene(s) underlying the differences in style length are likely to encode regulators of cell division rather than regulators of cell expansion.

The *P. exserta* and *P. a. axillaris N* accessions used in this study have been propagated in culture and are nearly homozygous. To determine whether they reflect the morphologies in wild populations, we compared them to a wild *P. exserta* population from the Serra do Sudeste region in Brazil and a wild *P. a. axillaris* population from José Ignacio, Uruguay. For *P. exserta*, we found similar pistil and stamen lengths for wild flowers and the inbred lines. For *P. a. axillaris*, slightly longer reproductive organs in the wild population compared to the inbred line were found (Fig. S5).

A major QTL on chromosome II affects reproductive organ length and floral tube morphology

We measured cell number and cell length in styles of *P. exserta* and *P. a. axillaris N* to study the cellular basis for the difference in pistil lengths. The overall average cell length was similar between the two species with $261 \pm 32 \mu\text{m}$ for *P. exserta* and $254 \pm 39 \mu\text{m}$ for *P. a. axillaris N*. In addition, no significant cell length variations were detected along the styles. However, a significant difference was observed for the average cell number: 139 ± 2 for *P. exserta* and 108 ± 7 for

A QTL analysis was performed for floral morphology traits in an F_2 population of a cross between *P. a. axillaris N* and *P. exserta* ($n = 203$) (Klahre et al. 2011). The F_2 progeny displayed pistil lengths from 3.83 ± 0.34 up to 5.42 ± 0.13 cm and dorsal stamen lengths from 3.75 ± 0.10 up to 5.41 ± 0.12 cm, spanning the entire parental phenotypic difference for these traits (phenotyping and genotyping data for all F_2 progenies are available at: <http://www.botany.unibe.ch/deve/research/projects/pollinator.php>). Similarly, a phenotypic distribution that covers the entire parental difference was also found for distal tube length (D2), stigma area, differences in stamen lengths and the distance between the dorsal stamens and the stigma. For proximal tube length (D1) and corolla limb size (D3), transgression was found with maximal values of 2.91 ± 0.12 and 3.04 ± 0.21 cm, surpassing both of the parental values by approximately one-third in both cases. For these two traits, transgression has been reported before

Fig. 2 Flower morphology of *P. exserta*, *P. a. axillaris* N and the F₁ hybrid. **a** Top view of *P. exserta*, *P. a. axillaris* N and F₁ hybrid flowers. **b** Side view of *P. exserta*, *P. a. axillaris* N and F₁ hybrid flowers. **c** Pistil and stamens lengths of *P. exserta*, *P. a. axillaris* N and F₁ hybrid flowers. From left to right: pistil, two dorsal, two lateral and one ventral stamen. Stamens were dissected from the floral tube above their fusion point. The white bar equals 1 cm



in an F₂ population derived from *P. a. axillaris* N and the closely related *P. axillaris parodii* S7 subspecies (Venail et al. 2010).

A number of QTLs were detected with individual effects explaining 5–50 % of the phenotypic variation explained (PVE) for each of these traits (Table 2; Fig. 3). A shared QTL was found on the top of chromosome II that influences male and female reproductive organ lengths as well as floral tube and limb size. It explains 42 % of the PVE for pistil length, 32–45 % of all three stamen lengths, 50 and 16 % of D2 and D1 tube lengths, respectively, and 41 % for D3 limb size. This QTL also accounts for the difference

in the distance between dorsal stamen and stigma. In all cases, it is supported by highly significant *P* values and LOD scores. Additional QTLs were found on chromosomes V and VII for pistil length and on chromosomes III and V for stamen lengths. Again, these loci were supported by highly significant *P* values and LOD scores. For stigma area, a major QTL was found on chromosome IV, explaining 24 % of the PVE and 3 minor QTLs on chromosomes I, III and IV. The difference in length between dorsal and ventral stamens can be attributed to a single QTL on chromosome VI (Table 2; Fig. 3). Thus, we found a major QTL for reproductive organ morphology on

chromosome II influencing the majority of the floral morphology traits we investigated and distinct QTLs on other chromosomes influencing individual traits.

In addition to the QTL analysis, the F_2 population was used to assess phenotypic correlations. The strongest correlations ($R^2 > 0.7$; Fig. 4) were found between stamen and pistil length and for dorsal, ventral and lateral stamen lengths (data not shown for ventral stamen lengths). Reproductive organ lengths were also correlated with floral tube length: stamen lengths with D1 and D2 tube lengths ($R^2 > 0.4$) and pistil length with D1 and D2 tube lengths ($R^2 > 0.5$) (Fig. 4). The strong phenotypic correlations can be explained by the shared QTL on top of chromosome II (Fig. 3). In accordance with this, only weak correlations were detected for all other traits.

The differences in reproductive organ morphology are specified by interactions between three loci

Considering that QTL effect sizes are negatively correlated with experimental sample sizes (Beavis 1994), our values may be inflated due to the moderate size ($n = 203$) of the F_2 progeny studied. To further investigate the QTL, we introgressed individual loci into reciprocal parental backgrounds and ‘mendelized’ the major QTL. Moreover, to study the genetic interactions between the different QTLs, we backcrossed informative F_2 lines and constructed reciprocal introgression lines (ILs) with combinations of QTLs. The pedigrees of all ILs are given in Fig. S1.

Introgressions into the *Petunia exserta* genetic background

We introgressed a 10 cM segment of chromosome II from *P. a. axillaris* N into the genetic background of *P. exserta* (IL4; crossing and mapping details are given in Fig. S1b), which was sufficient to explain the entire parental phenotypic difference for pistil length and most of the interspecific phenotypic difference for dorsal stamen length (Fig. S6). When homozygous, it caused pistil lengths of 4.06 ± 0.18 cm similar to the pistil length of the *P. a. axillaris* N parent (AA in Fig. 5a). Siblings that retained the introgression homozygous for *P. exserta* displayed pistil lengths similar to those of the *P. exserta* parent (5.41 ± 0.11 cm; EE in Fig. 5a). The heterozygous introgression resembled the *P. exserta* homozygous introgression (5.20 ± 0.13 cm; AE in Fig. 5a). This implies dominance of the *P. exserta* allele. Since *P. exserta* styles are longer because they have more cells (Fig. 1), we favor the hypothesis that a regulator of cell division with higher activity in *P. exserta* underlies the locus on chromosome II.

Three additional introgression lines were constructed that retained portions of the original introgression of IL4. IL5 still segregated for pistil length, indicating that this smaller introgression retains all genes required for extending pistils to the full *P. exserta* length (Fig. 5b). However, IL6 and IL7, two small non-overlapping introgressions contained within IL5 segregated for only minor effects on the pistil phenotype (Fig. 5c, d). The simplest interpretation of these data is that the QTL for pistil length is associated with the intervening marker MYB14. In this case, most of the parental difference could be explained by a small introgressed genomic region containing MYB14, which is flanked by the PAL1 and IGS markers. However, more complex scenarios with two interacting loci in IL6 and IL7 are equally plausible. The identification of the underlying gene(s) will be needed to elucidate the molecular nature of this QTL.

Could MYB14 be the causative gene underlying the QTL? Using semi-quantitative PCR and high-throughput transcriptome analysis, no differences in MYB14 expression could be observed (Fig. S7). However, a functional difference in *MYB14* cannot be excluded between *P. exserta* and *P. a. axillaris* N, due to two single nucleotide polymorphisms located in the ORF that translate into two amino acid changes, 51 and 180 amino acids after the R3 domain (Fig. S8).

In addition, we investigated the *Petunia* homologs of the *LO2* gene, which has been described to cause style elongation in tomato (Chen et al. 2007). We identified three *LO2* homologs in *Petunia*, named *LO2-01*, *LO2-02* and *LO2-03* (details can be found at <http://www.botany.unibe.ch/deve/caps/capslist.html>). One homolog (*LO2-02*) mapped to the QTL on chromosome II (Fig. S3), the other homologs mapped to chromosome IV (*LO2-01*) and to chromosome VI (*LO2-03*). Note that *LO2-02* is retained in IL6, which does not segregate for pistil length. We currently do not exclude *LO2-02* as a candidate gene as it might interact with a locus in IL7 to elongate the female reproductive organ (see above and Fig. 5).

Introgressions into the *Petunia a. axillaris* genetic background

When *P. a. axillaris* N was used as the recurrent parent, pistils were elongated by 0.53 ± 0.09 cm when the introgressed fragment of chromosome II was homozygous for *P. exserta* (Fig. 6a). Whilst in the *P. exserta* background, the QTL on chromosome II accounts for the entire parental difference of 1.33 ± 0.26 cm (Fig. 5), in the reciprocal introgression, this locus accounts only for $\sim 40\%$ of the parental difference. Therefore, additional loci must be required to elongate reproductive organs up to *P. exserta*

Table 2 QTL analysis

Trait	QTL	Chr	Position (cM)	CI (cM)	LOD	PVE	$P (=0.05) \chi^2$	Add	Dom
Pistil length	plQTL1	II	0.0	6	23.47	42	0.00000	0.37	0.16
	plQTL2	VII	31.3	14	9.07	19	0.00000	0.14	0.24
	plQTL3	V	27.7	21	5.79	13	0.00000	0.21	0.03
Lateral stamen length	lslQTL1	II	0.0	8	16.96	32	0.00000	0.18	0.10
	lslQTL2	V	28.6	9	15.40	30	0.00000	0.16	-0.09
	lslQTL3	III	42.1	24	5.14	11	0.00001	0.12	-0.02
Ventral stamen length	vsIQTL1	II	0.0	58	37.85	5	0.00000	0.33	0.11
	vsIQTL2	V	27.7	12	10.06	22	0.00000	0.21	-0.04
	vsIQTL3	III	42.1	18	6.96	15	0.00000	0.19	-0.00
Dorsal stamen length	dslQTL1	II	0.0	6	26.00	45	0.00000	0.23	0.10
	dslQTL2	V	27.7	13	10.06	21	0.00000	0.16	-0.05
	dslQTL3	III	55.9	28	4.34	9	0.00005	0.10	0.04
Difference between dorsal and lateral stamen length	ddslQTL1	VI	23.7	7	19.00	35	0.00000	-0.34	-0.22
D1 tube length	d1QTL1	VII	31.3	11	12.42	25	0.00000	0.12	0.11
	d1QTL2	III	42.1	17	7.44	16	0.00000	-0.13	-0.04
	d1QTL3	II	0.0	17	7.41	16	0.00000	0.12	0.11
	d1QTL4	I	48.4	17	7.33	15	0.00000	-0.14	0.01
D2 tube length	d2QTL1	II	0.0	5	30.36	50	0.00000	0.20	0.14
D3 limb length	d3QTL1	II	0.0	6	22.69	41	0.00000	0.25	0.22
	d3QTL2	IV	28.6	17	7.33	16	0.00000	-0.17	0.01
	d3QTL3	IV	0.0	19	6.51	14	0.00000	-0.15	0.06
Stigma area	saQTL1	IV	28.6	11	11.51	24	0.00000	-0.01	-0.00
	saQTL2	IV	0.0	16	7.74	17	0.00000	-0.00	0.00
	saQTL3	I	35.4	15	8.32	17	0.00000	-0.00	0.00
	saQTL4	III	32.7	20	6.16	13	0.00000	-0.00	-0.00
Distance dorsal stamen–stigma	ddssQTL1	I	53.2	18	6.91	15	0.00000	0.12	-0.07
	ddssQTL2	I	29.6	27	4.51	10	0.00001	0.11	-0.04
	ddssQTL3	II	0.8	22	5.68	12	0.00000	0.05	-0.07

LOD (logarithm of odds) is \log_{10} of the ratio of the probability that a QTL is present to the probability that a QTL is absent, *PVE* (phenotypic variance explained), the amount of the total trait variance which is explained by a QTL at this locus, expressed as percent, *CI* (confidence interval) estimate of 95 % confidence interval for a QTL of this strength, using the estimate of Darvasi and Soller (1994), *Add* the additive effect, *Dom* the dominant effect of the QTL

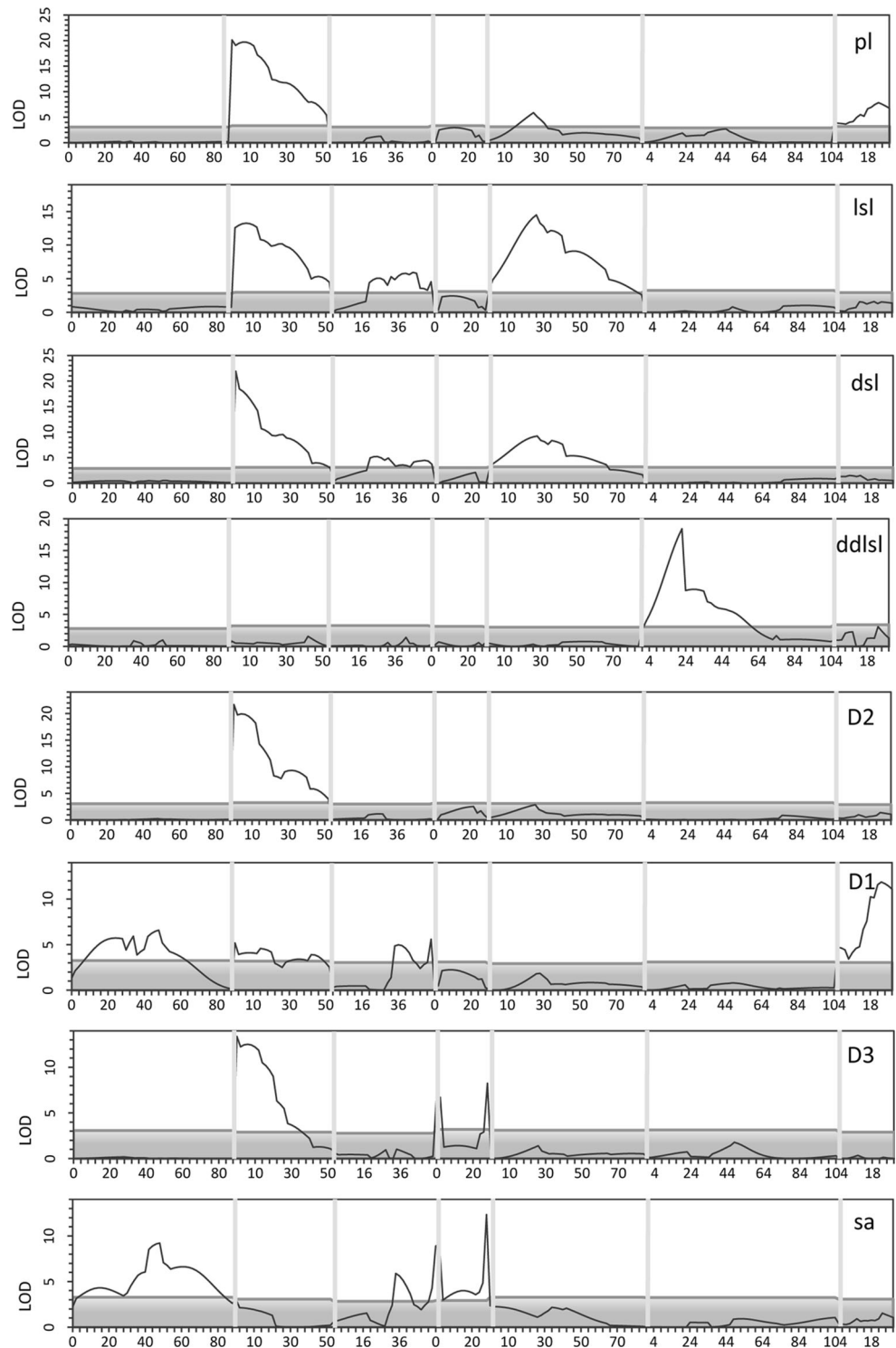
values. We identified two additional QTL for pistil length on chromosomes V and VII in the QTL analysis (Fig. 3). The single introgression of the chromosome V locus elongated pistils by 0.81 ± 0.18 cm (~ 60 % of the parental difference) and the single introgression of the chromosome VII QTL by 0.33 ± 0.08 cm (~ 25 % of the parental difference). Thus, each of these loci explains parts of the parental difference in pistil lengths (Fig. 6). Similar data were collected for stamen length (Fig. S9).

To test for additive or synergistic effects of these three loci, we bred introgression lines with multiple QTLs (Fig. 7). Co-introgression of the loci on chromosomes II and VII elongated pistils by 0.76 ± 0.10 cm, indicating

that these loci are almost fully additive and together explain 57 % of the difference between the parents (data not shown). The full *P. exserta* pistil length was recovered by the introgression of the loci on chromosomes II and V (Fig. 7). The combined effect of these two loci is the sum of their individual effects, thus they are fully additive. In this constellation, addition of the chromosome VII QTL had no effect, indicating that the chromosome VII QTL is redundant in the presence of chromosomes II and V (data not shown). Comparable results were obtained for stamen lengths (Fig. S10).

We propose a genetic model, in which the *P. exserta* alleles at all three loci are at least partially dominant. In the

Fig. 3 QTL analysis for reproductive organ and floral morphology. The y-axes indicate the LOD scores for each trait. Horizontal lines the significance threshold ($P < 0.01$) as determined by permutation analysis. The x-axes delimit the seven linkage groups; mapping distances are given in cM. *pl* pistil length, *lsl* lateral stamen length, *dsl* dorsal stamen length, *ddlsl* difference between dorsal and lateral stamen length, *sa* stigma area



P. axillaris background, the individual loci explain 40, 60 and 25 %, of the parental difference. The loci on chromosomes II and V are fully additive, whereas chromosome VII is redundant in the presence of chromosomes II and V (Fig. 8).

The chromosome II locus also impacts reproductive organ elongation in *Petunia axillaris parodii*

The *P. axillaris* subspecies, *P. a. parodii*, is similar to *P.a.axillaris* in all pollination syndrome traits except

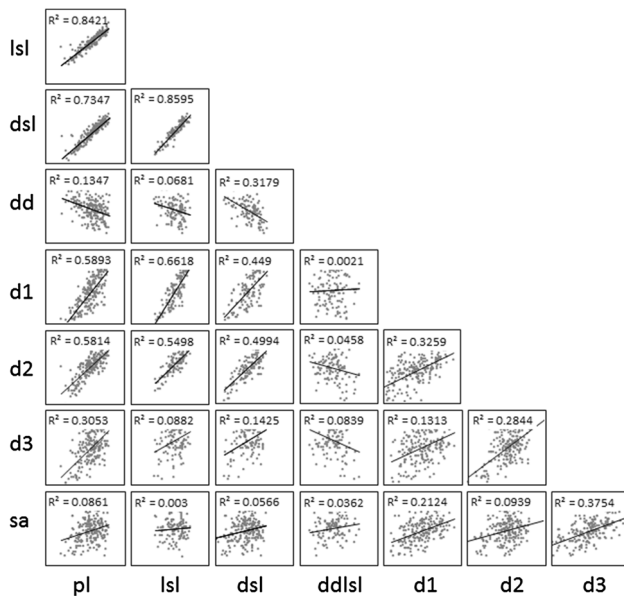


Fig. 4 Phenotypic correlations in the *P. exserta* × *P. a. axillaris* N F₂ population. Correlation plots of the indicated phenotypic parameters measured in the F₂ population. *pl* pistil length, *lsl* lateral stamen length, *dsl* dorsal stamen length, *ddsl* difference between dorsal and lateral stamen length, *sa* stigma area, *D1* proximal tube length, *D2* distal tube length, *D3* limb size

flower morphology. It also has white flowers but with longer reproductive organs and a longer corolla tube, compatible with pollination by long-tongued nocturnal hawkmoths, and a smaller limb size (Venail et al. 2010). An inbred accession of this subspecies, *P. a. parodii* S7, has a pistil length of 6.66 ± 0.34 cm, about 1 cm longer than *P. exserta* and about 2.5 cm longer than *P. a. axillaris*. In contrast to *P. exserta*, the simultaneous elongation of the floral tube causes the elongated reproductive organs to be non-exserted.

We asked whether the elongation of female reproductive organs in *P. a. parodii* S7 and *P. exserta* involves the same locus on chromosome II and introgressed the chromosome II loci of *P. exserta* and *P. a. axillaris* into the genetic background of *P. a. parodii* S7. The introgression of a chromosome II fragment from *P. exserta* into *P. a. parodii* S7 caused a shortening of the pistil length by 0.72 ± 0.17 cm compared to *P. a. parodii* S7 (~55 % of the parental difference). The heterozygous state of the same introgression led to pistils that were 0.35 ± 0.20 cm shorter.

The introgression of a chromosome II fragment from *P. a. axillaris* N into *P. a. parodii* S7 also caused a shortening of the pistil length. When homozygous, this introgressed fragment shortened pistil lengths by 1.04 ± 0.34 cm (~38 % of the parental difference) and in the heterozygous state by 0.61 ± 0.36 cm (Fig. 9). Again, the same pattern was observed for male reproductive

organs (Fig. S11). Thus, the locus on chromosome II is involved in the elongation of reproductive organs in *P. exserta* and *P. a. parodii* S7.

Discussion

Petunia exserta is thought to have evolved from *P. a. axillaris* in adaptation to a shift from hawkmoth to hummingbird-mediated pollination (Lorenz-Lemke et al. 2006). With respect to flower morphology, this involved the elongation of the corolla tube and an even stronger elongation of pistil and stamens. The short time elapsed since speciation invites the question of how the major morphological changes have occurred. We propose that the answer to this question lies in the surprisingly simple and modular genetic architecture that allowed for a rapid elongation of reproductive organs in adaptation to hummingbird pollination.

The QTL on chromosome II affects corolla tube, pistil and stamens. In contrast, the loci on chromosome V and VII affect fewer organs. The chromosome V QTL specifically affects the reproductive organs, whereas the locus on chromosome VII affects also proximal tube length. The QTL on chromosome II is furthermore shared between *P. exserta* and the *P. axillaris* subspecies *P. axillaris parodii*, two species with elongated reproductive organs. We speculate that the initial event may have been a change in the chromosome II locus, followed by genetic modifications in chromosomes V and chromosome VII with more specific phenotypic effects. Since the *P. exserta* alleles are at least partially dominant at all three loci, phenotypic effects will already be apparent as heterozygotes. Thus, the entire shift in floral organ elongation may have involved not more than three independent, (semi-) dominant mutations.

Large-effect genetic modifications may be atypical. It has been argued that they primarily involve simple biochemical traits (Rockman 2012). Indeed, functional polymorphisms of large effect have been well documented at the molecular level for floral color and floral scent (Bradshaw and Schemske 2003; Hoballah et al. 2007; Streisfeld and Rausher 2009; Hopkins and Rausher 2011) but for developmental traits such as organ length the situation is less clear. The modulation of organ length is a complex trait potentially involving a plethora of proteins regulating the cell cycle, microtubule organization, and anisotropic cell expansion (Berckmans and De Veylder 2009; Komaki and Sugimoto 2012). It cannot be excluded that our major QTL will fractionate upon fine mapping, or that major effects are due to a succession of small effect mutations in the same gene(s) (Studer and Doebley 2011; Martin and Orgogozo 2013). Definite proof one way or the other will

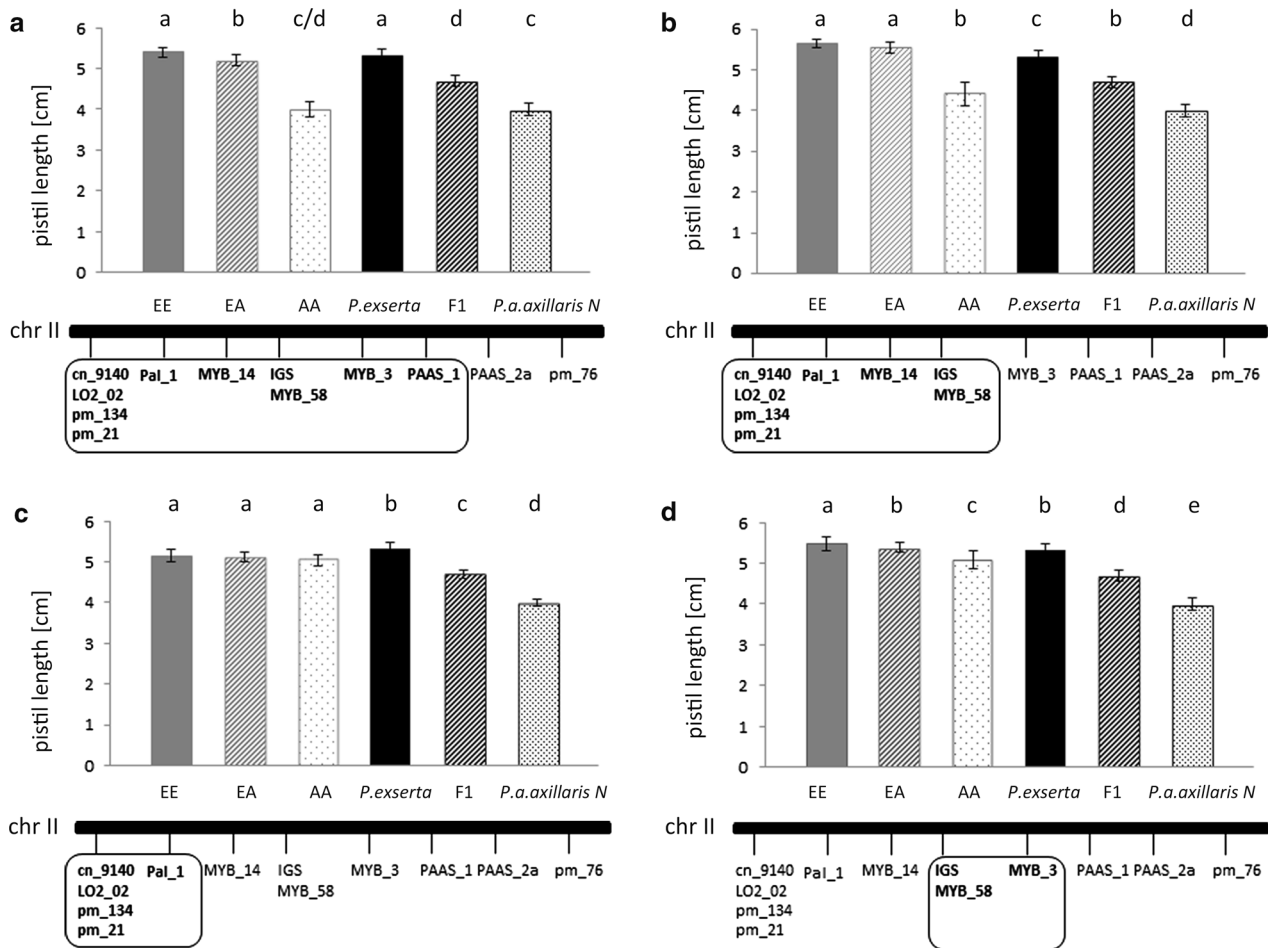


Fig. 5 The introgression of a part of chromosome II from *P. a. axillaris* N into *P. exserta* genetic background explains the entire phenotypic difference in pistil length. Pistil length measurements and genotypes are shown. *Boxed* molecular markers are from *P. a. axillaris* N and segregating. All other *markers* are homozygous for *P. exserta*. EE, homozygous for the *P. exserta* allele; EA, heterozygous; AA, homozygous for the *P. a. axillaris* N allele. *Marker intervals* are not to scale for better visualization, distances in

cM are shown in Fig. S3. *Error bars* show standard deviations, *different letters above bars* statistically different means ($P = 0.05$) as determined by the Mann–Whitney *U* test. **a** IL4: the segregation of the introgressed fragment explains the entire PVE in pistil length. **b** IL5: the segregation of the introgressed fragment explains most of the PVE in pistil length. **c** IL6: the introgressed fragment has a minor effect on pistil length. **d** IL7: the introgressed fragment has a minor effect on pistil length

require the identification of the underlying genes. The demonstration that the different style lengths of *P. a. axillaris* and *P. exserta* are caused by different cell numbers narrows the search for candidate genes to regulators of cell division.

From a system’s biology perspective, there is no strong justification to discriminate between simple biochemical and complex morphological traits. We argue that changing a “simple” biochemical trait such as color or scent is not simple. Anthocyanins derive from the common amino acid phenylalanine, which is also the precursor of lignins, phytoalexins, benzenoid volatiles and flavonols. Anthocyanins and flavonols are not only important pollinator attractants, but they also serve as defense compounds and regulators of polar auxin transport, respectively (Jacobs

and Rubery 1988; Mo et al. 1992; Li et al. 1993; Shirley 1996; Napoli et al. 1999; Brown et al. 2001). Such metabolic and regulatory crosstalk between pathways puts severe constraints on network flexibility. Conversely, major changes in complex morphological traits can happen through few mutations of major effect. Some 80 % of the morphological differences between maize and its wild ancestor *Teosinte parviglumis* can be traced back to five major genes (Doebley 2004). In the case of the branching gene *teosinte branched*, naturally occurring alleles of similar phenotypic magnitude were found in wild *Teosinte* populations (Studer and Doebley 2012).

The large-effect scenario is thought to be most plausible when a genotype is displaced far from its adaptive optimum (Rockman 2012). Changes in pollinator

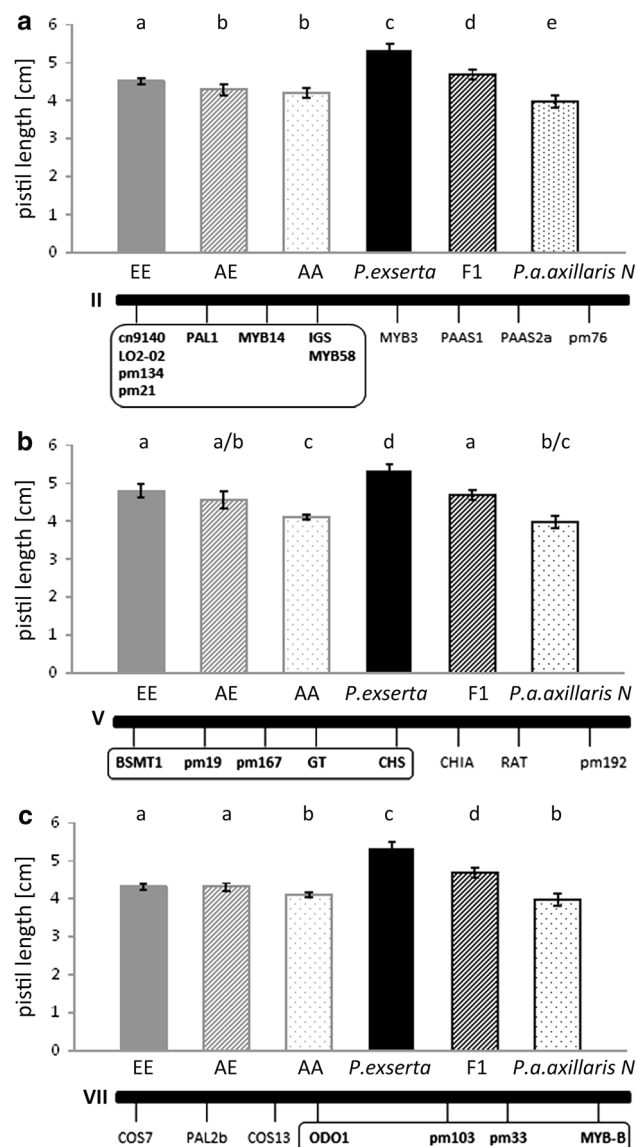


Fig. 6 The single introgressions of the QTL on chromosomes II, V and VII from *P. exserta* into *P. a. axillaris* N have different effects on the elongation of reproductive organs. Pistil length measurements are shown. *Boxed* molecular markers are introgressed from *P. exserta* and segregating. *Marker* intervals are not to scale for better visualization. *Error bars* show standard deviations, *different letters* statistically different means ($P = 0.05$) as determined by the Mann–Whitney U test. **a** IL8: Introgression the QTL on chromosome II explains ~40 % PVE in pistil length. **b** IL9: introgression of the QTL on chromosome V explains ~60 % PVE in pistil length. **c** IL10: introgression of the QTL on chromosome VII explains ~25 % PVE in pistil length

availability due to disease or invasion of competing plant species may occur rapidly and frequently. Under such circumstances, the gradual accumulation over evolutionary time of multiple small mutations may well be too slow to secure survival. Recently, we found tight genetic linkage between the chromosome II locus shaping male

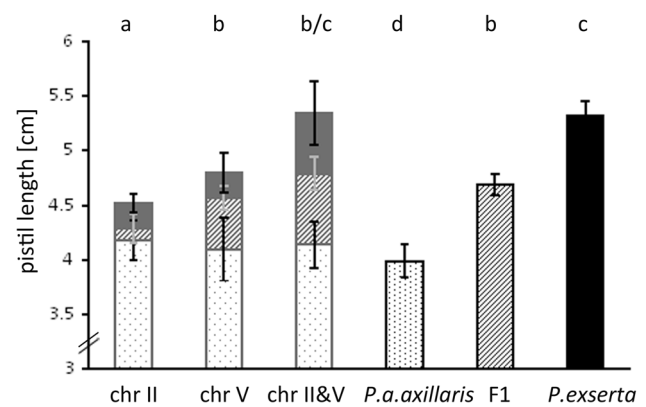


Fig. 7 The introgression of two QTLs on chromosomes II and V is sufficient to explain the entire interspecific difference in reproductive organ morphology. The allelic effect on pistil elongation is shown for the single introgression of chromosomes II and V and the multiple introgressions of both loci. Pistil lengths for the siblings that retained the introgression homozygous for *P. a. axillaris* N are displayed by the spotted pattern. The gains in pistil length that are obtained by the heterozygous introgressions (for both loci in the multiple introgressions) are shown by the hatched pattern and the ones obtained by the homozygous *P. exserta* introgressions (also for both loci in the multiple introgressions) are shown in gray. *Error bars* show standard deviations, *different letters* statistically different means ($P = 0.05$) of the homozygous *P. exserta* introgressions as determined by the Mann–Whitney U test

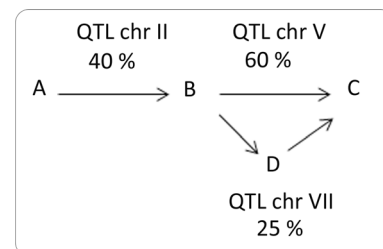


Fig. 8 A genetic model for the interaction of the QTL on chromosomes II, V and VII shaping reproductive organ morphology in the genetic background of *P. a. axillaris* N. The *P. exserta* allele is at least partially dominant at all three loci, and all loci have an additive effect on the elongation of reproductive organs. The chromosome VII locus acts redundantly to the chromosome V locus

and female reproductive organ morphology and loci specifying floral scent production, UV absorption, and visible color (Hermann et al. 2013). Irrespective of the number and individual effect size of the underlying genetic modifications, such tight genetic linkage would allow hybridizing populations to shift between pollinators within a few generations.

In addition to dissecting the genetic basis of differences in reproductive organ morphology, our introgression lines provide excellent material to test for the effect of style and stamen exertion on pollen transfer and pollination efficiency due to hummingbird or hawkmoth pollination. Substitutions of individual traits in defined genetic

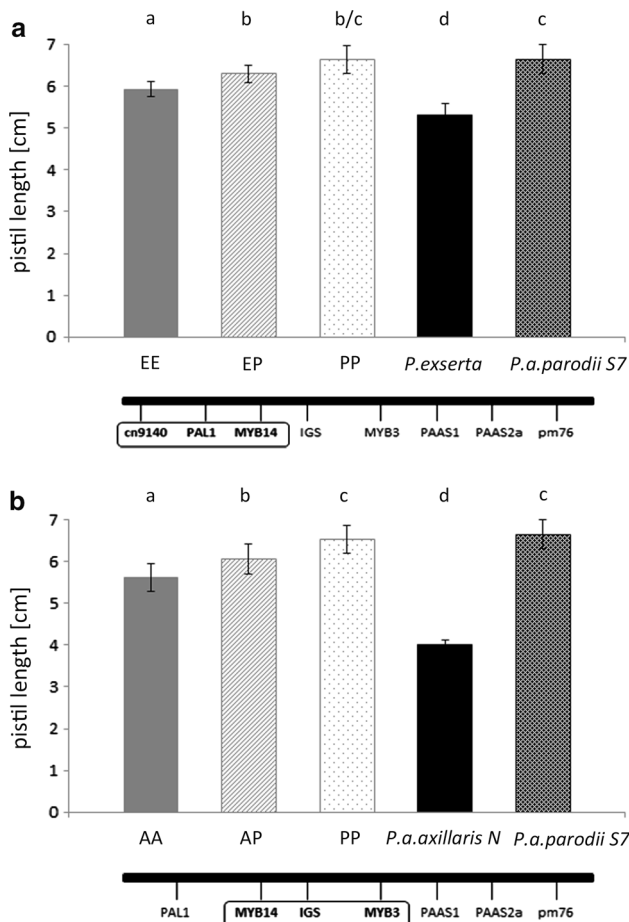


Fig. 9 The introgressions of the chromosome II QTL from *P. exserta* and *P. a. axillaris N* into *P. a. parodii S7* shorten pistil lengths. Pistil length measurements are shown. Boxed molecular markers are introgressed and segregating. Error bars show standard deviations, letters statistically different means ($P = 0.05$) as determined by the Mann–Whitney U test. **a** IL14: the introgression of chromosome II from *P. exserta* causes significantly shorter pistils compared to *P. a. parodii S7*. **b** IL15: the introgression of chromosome II from *P. a. axillaris N* causes significantly shorter pistils compared to *P. a. parodii S7*

backgrounds will help to determine whether the exertion of reproductive organs is actually an adaptation to increase pollination efficiency by hummingbirds. Such experiments are a key step to unravel the importance of reproductive organ morphology for pollination success and therefore reproductive isolation in animal-pollinated plants.

Author contribution K.H, U.K. and C.K. designed the research, K.H. and U.K. conducted the research and analyzed the data, J.V. contributed novel biological materials and A.B. helped with the statistics. K.H. and C.K. wrote the article.

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Conflict of interest The authors declare that they have no conflict of interest.

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