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The genetic dissection of floral pollination syndromes

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A major factor in the evolution of the angiosperms is the adaptation of plants to animal pollinators. The specific morphology of a flower, its color, nectar composition and scent production can all contribute to reproductive success by attracting pollinators and by limiting out-crossing with other species. It has now become feasible to dissect the genetic basis of plant adaptation to different pollinators.

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Current Opinion in Plant Biology 2006, **9**:78–82

This review comes from a themed issue on
Growth and development
Edited by David Smyth and Thomas Berleth

Available online 1st December 2005

1369-5266/$ – see front matter

DOI 10.1016/j.pbi.2005.11.003

Introduction

What constitutes a species and what is the genetic basis of speciation is a field of endless controversy but surprisingly few identified genes. Higher plants offer important practical advantages for the study of speciation genetics. First, the opportunity to perform wide interspecific crosses allows the functional genetic analysis of a large variety of taxonomic traits. This compares favorably with most animals, in which strong reproductive barriers preclude interspecific crosses except in closely related special cases [1–4]. Second, the sessile nature of plants makes it feasible to study selected offspring of interspecific crosses in the field, and thereby assess the phenotypic effect of identified genes under natural conditions.

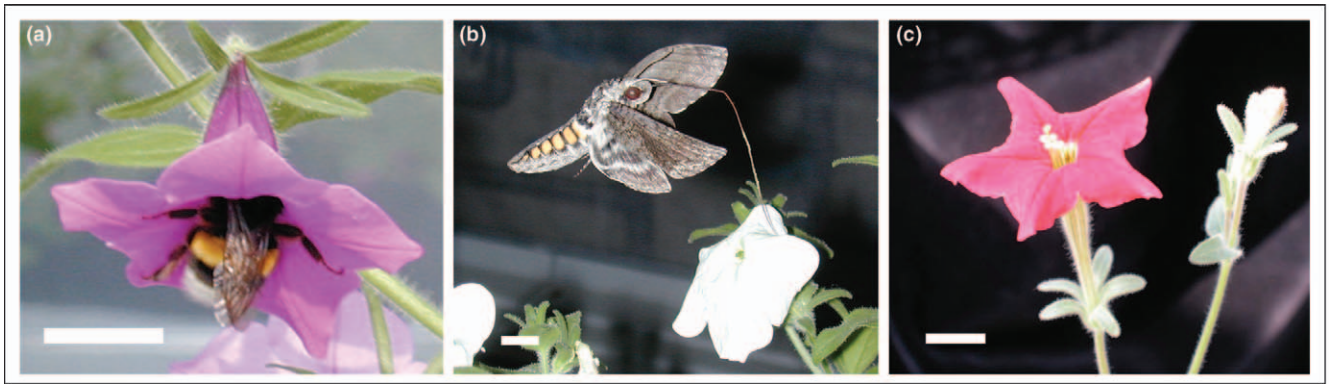
Numerous plant species rely on animal pollinators for their reproduction and have evolved with them through directional selection towards a complex of phenotypes that enhance reproductive success, such as floral architecture, color, scent and nectar. ''Regardless of their taxonomic relationship, flowers pollinated by particular visitors tend to show particular features in common, related to the size, behavior and other biological characteristics of their pollinators... These patterns of common characters, to which flowers of quite different evolutionary origins may converge, have been called pollination syndromes'' [5]. In most cases, it remains unclear how the individual floral features contribute to pollinator behavior, or how combinations of these features are integrated by the animal's sensory system. With such complex combinatorial possibilities, the difficulty lies in assessing precisely the role of single floral features in reproductive isolation. One of the recent themes in pollination biology has been to genetically disentangle pollination syndromes into their component traits (i.e. color, scent, shape, nectar and so on) and to study them individually in defined genetic backgrounds. Genetic and ecological studies might then answer how pollinator shifts are conditioned by natural allelic variation in floral traits. One of the great strengths of this approach is that it might quantify the level of isolation of groups of plants that result from shifts in pollinator visitation. We review what is known about the genetic basis of adaptation in the case of pollinator-mediated selection, emphasizing more intensely studied systems such as *Mimulus*, *Antirrhinum*, *Ipomoea*, *Clarkia* and finally *Petunia*, our own model species.

Morphology

Bilateral symmetry has been proposed as a mechanism to facilitate pollen transfer by insects that are themselves bilaterally symmetric. For example, the mechanics of pollen deposition onto the bumblebee body by many species of *Lamiales* (e.g. *Antirrhinum*) depends on a bilaterally symmetric floral body plan [5]. The flower shape, and especially the floral bilateral symmetry, of *Antirrhinum majus* has been extensively studied. Several genes that are needed for bilateral symmetry have been identified, such as *CYCLOIDEA* (*CYC*) and its paralog *DICHOTOMA* (*DICH*). (These two genes encode TCP-related transcription factors that are similar to that encoded by the maize gene *teosinte branched 1* [6–8].) *CYC* and *DICH* are expressed at early stages of floral development, but their expression patterns differ between *A. majus* and the closely related *Mohavea confertiflora*, a species that has radial symmetry [9]. It is possible that these differences in expression patterns account for the different floral morphologies of the two species [9]. Although *A. majus* and *M. confertiflora* are visited by different bee-pollinators it is difficult to correlate floral symmetry and pollinator preference as the two plant species are strongly diverged and belong to two different genera.

Over the years, Bradshaw and Schemske [10,11] have explored the ecology and genetics of pollination syndromes in the two monkeyflower species *Mimulus lewisii* and *Mimulus cardinalis* in great depth. These species display divergent pollination syndromes that are specific

Figure 1

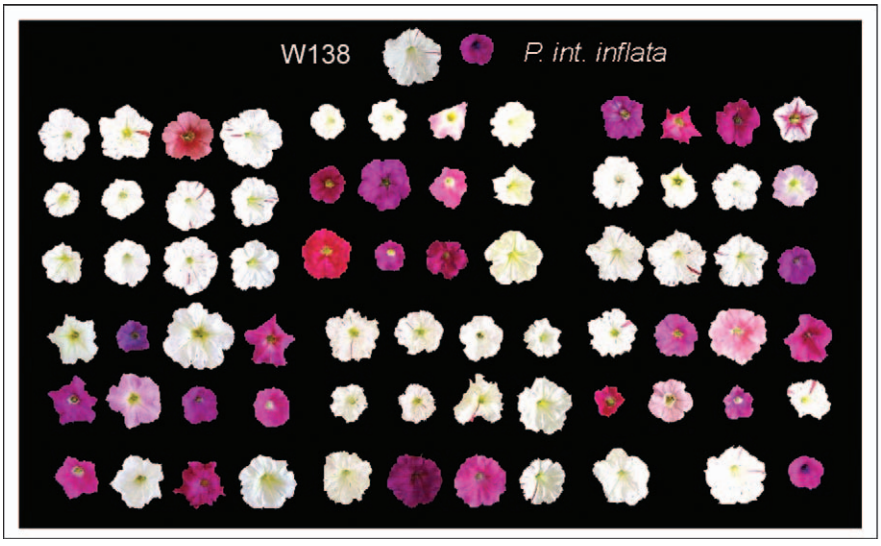


Petunia ecotypes and their pollinators. **(a)** *Petunia integrifolia* visited by the bumble bee *Bombus terrestris*. **(b)** *Petunia axillaris* visited by *Manduca sexta*. **(c)** The rare species *Petunia exserta*, which is adapted to hummingbird visitation. The scale bar represents 1 cm. All images were taken in the greenhouses of the Institute of Plant Sciences, Berne. (a) Provided by Maria-Elena Hoballah.

for bumblebees and hummingbirds, respectively. Using an approach that was based on quantitative trait loci (QTL), Bradshaw and Schemske [10,11] mapped several loci that control differences in floral color, shape and size and nectar volume. For each of these traits, the authors found at least one QTL of 'large effect' (i.e. accounting for more than 25% of the phenotypic variation explained [PVE]). Regarding flower morphology, multiple QTL (4–8 per trait) were detected for petal and corolla width, corolla projected area and aperture and petal reflexing [10,11]. It is not yet known, whether these loci comprise single or multiple genes. In addition, it remains unclear how this level of PVE translates into absolute differences in shape between parental genotypes. To resolve such issues, nearly isogenic lines (NILs) will have to be developed at high genetic resolution and the genes involved isolated.

We performed a similar analysis in our model system *Petunia*. *Petunia* has excellent forward and reverse genetics that provide a major advantage in the dissection of complex traits, including pollination syndromes [12••,13]. The genus contains three distinct pollination syndromes, with *Petunia integrifolia*, *Petunia axillaris* and *Petunia exserta* adapted to bees, hawk moths and birds, respectively (Figure 1). The flowers of each of these three *Petunia* species has a distinct morphology. *P. axillaris* has large flowers that have a long tube, whereas *P. integrifolia* has small flowers with a short and broad tube. The intensely red *P. exserta* has the exserted stamens and pistil that are typical of bird-pollinated flowers (Figure 1). A QTL study of recombinant inbred lines (RILs) (Figure 2) involving *P. integrifolia* and *P. axillaris* has allowed the genetic dissection of the pollination syn-

Figure 2



Recombinant inbred lines (RILs) of *Petunia hybrida* W138 and *Petunia int. inflata* (S6) [12••]. Flower traits segregate widely in the BC1F5 generation.

dromes into their individual traits. Floral-tube morphology appears to be controlled by at least five loci ([12••]; C Galliot *et al*., unpublished) of small to moderate effect. This number is likely to be an underestimate because of modest map resolution. One could hypothesize that, at least in *Petunia*, floral-tube morphology has adapted gradually to optimize pollen transfer by distinct insect visitors, which might have preferred the flowers for reasons other than tube morphology.

No explicit experiments have been performed to test whether bilaterally symmetric *Antirrhinum* mutants, or NILs for morphology QTLs in *Mimulus* or *Petunia*, attract pollinators differentially or to what extent they might affect pollen carry-over. As the required genetic material is becoming available, there are now excellent possibilities in this direction, with the added value that the model systems that are being used involve several different pollination systems.

Color

In *Mimulus*, a QTL of large effect was detected for petal carotenoid content. It contains the *YELLOW UPPER* locus (*YUP*), which segregates in a Mendelian fashion. Lines with reciprocal introgressions of the *YUP* locus were constructed and pollinator behavior was assessed in the natural habitat. When introgressed into the *M. lewisii* genetic environment, the *M. cardinalis yup* allele dramatically increased visitation rate of hummingbirds and simultaneously lowered that of bees [14••]. The ratio of bees' to hummingbirds' visitations dropped from over 700:1 in the wildtype to 1.8:1 in a NIL that housed this locus. Bradshaw and Schemske [14••] suggest that a small decrease in bee population would provide the appropriate circumstance for the *yup* allele to be positively selected. *YUP* has not yet been resolved as a single gene and the molecular identity of this QTL remains unknown.

The anthocyanin biosynthesis pathway has been well studied in *Petunia*, *Antirrhinum* and maize, in which most of the biosynthetic enzymes and regulatory loci have been identified [15]. Anthocyanin-2 (AN2) is a MYB-domain transcription factor that is specifically expressed in the petal limb and that regulates the expression of the late enzymes. Genetic crosses between bee- and hawkmoth-pollinated *Petunia* indicated that functional polymorphism in AN2 can explain most of the petal limb color difference between the purple *P. integrifolia* and the white *P. axillaris* [16,17]. A key question will be how reciprocal transfer of *AN2* alleles between the two species will impact pollinator preference. Sequencing of this gene in several garden *Petunia* and in wild accessions revealed that multiple loss-of-function alleles arose independently [17]. The phylogenetic data on *AN2* strongly suggest that the loss of color in *P. axillaris* occurred after the split of *P. integrifolia* and *P. axillaris*. Therefore, other events might have preceded the loss of *AN2* activity and separated the two species [17].

In *A. majus*, the MYB-related transcription factor MIXTA is required for the formation of conical cells on the inner epidermis of the petals [18]. Although *mixta* mutants are not impaired in pigmentation itself, their flowers display a dull light-magenta color, which is the result of an abnormally low absorbance of light. A field study highlighted that pollinator visitations were strongly correlated with the presence of conical cells, even more so than to pigmentation *per se* [19]. *MIXTA* is not polymorphic between closely related species and there is no direct evidence that it contributes to reproductive isolation. Conical cells, together with bilateral symmetry, might have evolved early in specialized pollination by bees [20••].

Ipomoea purpurea, the common 'morning glory', is highly polymorphic for flower color and the pathways leading to the different pigment production are well known [21]. One gene, *flavonoid-3′-hydroxylase* (*F3′H*), controls whether cyanidin (blue) or pelargonidin (red) pigments are produced. Zufall and Rausher [22••] indicate that the inactivation of the cyanidin pathway in *Ipomoea quamoclit*, a red subspecies that is thought to derive from a blue/purple ancestor, is caused by the downregulation of the enzyme F3′H. In addition, *in vitro* assays indicate that dihydroflavonol 4-reductase (DFR), which acts downstream of F3′H, seems to have lost its substrate affinity in *I. quamoclit*. Although *Ipomoea* has the advantage of well-studied color genes, these species were bred and selected for their color by pre-Columbian people, and therefore, their genetic polymorphisms might be the result of man-made rather than natural selection [23].

Scent

Volatiles serve as short- and long-distance cues for pollinators, in particular for attracting nocturnally active moths [24]. *Clarkia breweri* has been used as a model for studying the evolution of floral scent. The strong, sweet floral scent of *C. breweri* flowers has linalool as a major constituent, which is unique in this genus. Scent in *C. breweri* is correlated with pollination by moths, a mode of reproduction that is novel among *Clarkia* species [25,26]. The expression pattern of the *S-linalool synthase* (*Lis*) gene, which encodes a terpene synthase, is different in *C. breweri* than in other *Clarkia* species. Not only is *Lis* expression in the stigma of *C. breweri* greater than that in other *Clarkia* species but *Lis* is also expressed in other floral tissues of *C. breweri*; for example, in the petals, a major site of odor production [27]. Therefore, the evolution of scent in this system involved the recruitment of an existing gene, with expansion of its expression pattern underlying the functional difference. It would be of particular interest to test pollinator attraction to NILs that differ in *Lis* expression, and to determine whether it can cause reproductive isolation. The fact that the *C.*

breweri pollination syndrome is unique in its genus precludes a phylogenetic approach to *Lis* evolution and its role in speciation. The progress made in *Clarkia* has, however, served to further identify scent genes in snapdragon and *Petunia* [28,29].

In addition to terpenoids, phenylpropanoids/benzenoid compounds and volatile fatty-acid derivatives are major components of floral scent [30]. *Petunia axillaris* emits high levels of benzenoid compounds (i.e. benzaldehyde, benzyl alcohol and methyl benzoate) in a strict circadian rhythm that matches the behavioral activity of the moth pollinators. By contrast, volatile production in the bee-pollinated *P. integrifolia* is minor [31••]. The produced benzenoids are derivatives of a common precursor, benzoic acid [32], and their production depends on enzymes such as benzoic acid salicylic acid methyltransferase (BSMT) [33] and a benzoyl-CoA: benzyl alcohol/phenylethanol benzoyltranferase (BPBT), which are specifically expressed in the petal limbs [34••]. Furthermore, the expression levels and/or the activity of these two enzymes follows a circadian rhythm. One recently cloned gene, *ODORANT1*, which encodes a MYB-type transcription factor, regulates the synthesis of benzenoid compounds in the petals by producing precursors in the shikimate pathway. This gene seems to control, at least in part, the rhythm of odor production in *P. axillaris* [35••]. With 'floral scent genes' being identified rapidly, it will soon be feasible to identify functional polymorphisms in scent production between the species by candidate gene approaches. These genes can then be used in defined genetic backgrounds to study their effects on pollinator preference.

Nectar

The genetic control of nectar production and how it contributes to the differences in nectar volume and nectar composition among ecotypes or species is poorly understood. In *Mimulus* and *Petunia*, a minimum of two QTLs are involved in controlling the amount of nectar produced [10,12••]. In both cases, one of these QTLs accounts for one-third of the parental difference. The hexose:sucrose ratio of the nectar in *Petunia* is under the control of a major QTL, which might correspond to the activity of an invertase [12••]. Nectar QTL are notoriously difficult to study because of the large environmental variation in this trait. Much more effort will be needed to arrive at the reliable identification of the genes involved. Nectar is the major reward offered to pollinators and, therefore, lines that differ exclusively in nectar content or composition will be invaluable tools for studies of insect behavior.

Conclusions

We have summarized the progress made in the genetic dissection of a complex trait, the pollination syndrome. In several cases, QTL mapping suggests the presence of individual loci of moderate to strong effect. This is reminiscent of the situation in maize, where five loci account for most of the phenotypic difference with its predecessor *teosinte* [6]. One should bear in mind, however, that in the cases reviewed here, the mapping populations have generally been small and the genetic maps of only moderate resolution. It is possible that the five major maize QTL might indeed correspond to five genes, whereas it is entirely conceivable that more-detailed efforts will resolve some of the large 'pollination' QTL into multiple loci of smaller effect.

A major question for future research will be whether the identified QTL represent speciation genes. 'Large-effect QTL' are likely to induce rapid changes that lead to abrupt shifts in pollinator preference. Although they might have contributed to reproductive isolation, the evidence that these loci are the primary cause of reproductive isolation is weak. Despite the appeal of floral 'insect-traits' as isolating mechanisms, we emphasize that other mechanisms, such as gametic incompatibility, hybrid inviability or sterility, should be further investigated as they could also contribute to speciation [2].

Genetic dissection of pollination syndromes into their individual components will make it possible to study the effect of defined genetic polymorphisms on pollination behavior, separately and in combinations. This will help elucidate the role of individual genes in the evolution of pollination syndromes, in reproductive isolation and possibly in speciation.

Acknowledgements

We would like to thank Maria-Elena Hoballah and Kath Bainbridge for critical reading of the manuscript, and D Wittmann for sharing unpublished information on *Petunia exserta*.

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