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Heteromorphy and Flower Development in *Primula*

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ABSTRACT

The phenomenon of heterostyly, the possession of floral morphs with differing style lengths within the same plant species, occurs across 28 plant families. One hundred and forty years after its first description in the Primulaceae by Darwin, heterostyly has been studied extensively, but is still not fully understood. Heterostyly functions to maintain sexual diversity by preventing self-fertilization. *Primula* is distylous, with flowers existing in two forms, "pin" and "thrum," each having reciprocal placement of the anthers and stigma. Several other morphological differences – such as pollen number and size, stigmatic papillae, and stylar cell length – occur between the two forms, as well as genetic differences of the traits, with pin being homozygous recessive (*ss*) and thrum heterozygous (*Ss*). Research on heterostyly in *Primula* has focused largely on the anatomical and physiological differences between the two floral morphs, and traditional genetic studies of the self-incompatibility (*S*-) locus that governs this breeding system. The *S*-locus has been shown to be a "supergene complex" of genes held in linkage disequilibrium and inherited as a single unit. Developing a thorough understanding of this breeding system promises to have a broad impact not only on the knowledge base of the sexual incompatibility systems of plants, but also on the applied industries of agriculture and horticulture. In this article we provide an overview of our current understanding of this system.

Keywords: floral development, self-incompatibility, *Primula*

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INTRODUCTION

Heterostyly is a genetically controlled floral polymorphism that results in either two (distyly), or three (tristyly) floral morphs in plants of a single species, each individual bearing flowers of just a single morph. The term itself was coined by Hildebrand and popularized by Charles Darwin, who used this term in "The Different Forms of Flowers on Plants of the Same Species" (Darwin 1877). To date heterostyly has been noted in 28 plant families (Barrett 2002), which are phylogenetically scattered throughout the Angiosperms, leading to the belief that these systems have evolved independently, though they have done so with remarkable consistency. The floral morphs differ grossly in that they exhibit reciprocal positioning of anthers and stigmas, but additional floral polymorphisms are often present as well as genetic linkage of these traits to diallelic, sporophytically controlled self-incompatibility, to form a heteromorphic self-incompatibility (HSI) system. While HSI systems can be dimorphic or trimorphic, *Primula* exemplifies the dimorphic condition. Plants with anthers positioned above the stigma are termed "thrum" while those with stigmas higher than the anthers are termed "pin" (**Fig. 1**). Darwin's studies not only described the physical differences between the floral morphs, but also demonstrated that these differences promoted out-crossing, and led to HSI in *Primula* becoming a major research model for botanists and geneticists for much of the first half of the twentieth century.

Classical genetic studies on HSI, and *Primula* in particular, abounded in first half the 20th century. These systems represent a remarkable example of convergent evolution in floral morphology, genetics and physiology, and have attracted considerable attention as model systems to study the

Fig. 1 Pin (A) and thrum (B) floral morphs of *Primula vulgaris*. Anthers and stigma are reciprocally positioned between the morphs.

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Invited Mini-Review

Table 1 Floral polymorphisms between pin and thrum flowers in *Primula*.

Character	Polymorphism
style length	approximately reciprocal to stamen length
style cell size and shape	larger cells in the pin morph
overall stigma shape	flatter in thrums
length of stigmatic papillae	longer in pins
stamen length	see style length
pollen size	~50% smaller in volume in pin
pollen number	2-3 fold more numerous in pin
corolla tube mouth	wider in thrums

evolution, genetics and adaptive significance of plant reproductive systems (Ganders 1979). Yet since the rise of *Arabidopsis* and *Antirrhinum*, the attention of both geneticists and developmental biologists has been focused elsewhere. As a result very little is known about the molecular regulation of heterostyly. Molecular characterization of these breeding systems promises to significantly enhance our understanding of floral development, in particular the regulation of organ size and positioning. Whilst still in their early stages, recent efforts have begun which promise to help rectify this situation and enable the integration of molecular data with classical genetics and dramatically improve our understanding of how these intriguing breeding systems operate.

MORPHOLOGY

HSI has been noted in 388 species of the genus *Primula* (Al Wadi and Richards 1993). As well as differing style lengths *Primula* also exhibits a number of additional polymorphisms between the two floral morphs, unlike some other heterostylous species. **Table 1** shows the characters that differ between pin and thrum morphs in *Primula* species (modified from Richards 1986), all are under the genetic control of factors linked to the *S*-locus.

Given the nature of these polymorphisms, a number of which appear to be related to organ or cell size, it would seem intuitively likely that multiple characters might be controlled by a single regulatory gene. As described below however, analysis of rare recombinants within the *S*-locus as well as recent characterization of the developmental profiles of the two morphs suggest that there are in fact separate genes and developmental pathways controlling many of these characters. These morphological features play a distinct role in self-incompatibility of distylous plants and represent co-adapted traits which act in concert to both reduce intra-morph pollination and to promote inter-morph pollination (Dulberger 1992); for example, the large pollen of the thrum plants is it better suited for pollinating the long papillae of the pin flower's long stigmatic papillae and *vice versa*. Pollination of *Primula* species is primarily effected by Hymenopterans, but Lepidopterans, Coleopterans and Dipterans also participate. Each species emits a specific floral odor, reducing interspecific hybridization. Between morphs of a particular species, however, floral scent shows little or no variation leading to a randomization of pollinator preference and ensures that both pin and thrum plants are visited (Gaskett *et al.* 2005).

Some *Primula* species, as well as mutants of normally heterostylous species, exhibit either a short or long homostyle flowers. In these cases, the plants are self-fertile. There has been considerable debate over whether any of the homostylous species represent the ancestral state or whether all have evolved from heterostylous ancestors. Recent work suggests the latter, i.e. that these species have experienced a recombination within the linkage group that controls the mating system such that a single plant has thrum-type pollen with pin-type stigmas or *vice versa* (Mast *et al.* 2006).

The function of heteromorphic traits

Darwin's experiments led to the conclusion that heterostyly was an elegant outbreeding mechanism (1877). Ultimately these breeding systems act to prevent self- and promote cross-pollination. HSI renders *Primula* not only incapable of self-fertilization, but also nearly incapable of fertilization within the same morph. Darwin's experiments demonstrated that compatibility between the morphs of distylous flowers must be of heights along the same plane (Darwin 1877). For example, pollen from a thrum flower was compatible with a stigma from a pin flower and pollen from a pin flowers was compatible with a stigma from a thrum flowers. Crosses between pin and thrum plants and thrum and pin plants revealed nearly equal numbers of pin and thrum progeny (Darwin 1862). Positioning alone, however, is not the only cause for compatibility. Pollen size, associated with the floral morph, also contributes as well as a biochemical incompatibility system.

A considerable number of theories have been proposed as to the function of the stamen/style polymorphism. One hypothesis is that morphological characters are an integral part of the self-incompatibility system at the physiological level (Dulberger 1975). An alternative view is that the various morphological polymorphisms each have functions of their own, and are not merely a morphological manifestation of the SI response (Ganders 1979). The fact that HSI appears to have evolved independently with remarkable consistency suggests that whether or not morphological traits play a prime role in self-incompatibility *per se*, they must play an important role in this breeding system overall. To date it has been impossible to assay the significance of each character in isolation. Identification and manipulation of the individual components of the linkage group through the use of transgenic plants would be a valuable tool in resolving these issues.

GENETICS OF THE HSI *S*-LOCUS

The self-incompatibility (*S*)-locus in *Primula* is hypothesized to be a "supergene complex", which has two functional alleles. The study of this supergene complex in *Primula* has a long and impressive history, having attracted the attention of many of the leading evolutionary biologists and geneticists of the 19th and 20th centuries, including Darwin (1862, 1877), Bateson and Gregory (1905), Ernst (1936), Haldane (1938), Mather (1950), Lewis (1954), Ford (1964) and Darlington (1971) have contributed to the subject.

The two alleles of the HSI *S*-locus act sporophytically, one showing complete dominance over the other (Mather 1950; Dowrick 1956). The thrum morph is heterozygous (*Ss*) whereas the pin morph is homozygous recessive (*ss*) (Bateson and Gregory 1905; Mather 1950). The genes controlling biochemical self-incompatibility are tightly linked to the genes controlling floral polymorphism and for the most part, segregate as a single genetic unit. Rare crossover events do occur within the locus, however, and give rise to self-compatible, homostylous plants.

Based on work spanning almost a century, the various features of HSI in *Primula* appear to be controlled by one, or in some cases more than one, unique genetic component within the *S*-locus. Studies of recombinant homostyles have led to the identification of a number of sub-units within the *S* linkage group and the development of a letter naming system associated with particular morphological features (Ernst 1936). *G* (female traits including style length, stigmatic papilla type, and female incompatibility), *P* (pollen traits: pollen size, male incompatibility) and *A* (anther height) (Ernst 1955; Dowrick 1956). Over the years, there has been considerable debate about the order in which these genetic components occur in the *S*-locus. The accepted order (*GPA*) was established by Dowrick (1956) and confirmed by Lewis and Jones (1992), with the understanding that the recombination events may depend upon the particular taxa involved. According to this designation the thrum form is *GPA/gpa* and

Table 2 Sub-components identified in the *Primula S*-locus.

Sub-component	Trait
1. G/g	female incompatibility phenotype and style length (in part)
2. Mpm/mpm	sporophytic dominance for pollen size (position uncertain)
3. Pp/pp	pollen size (gametophytically)
4. Pm/pm	male incompatibility phenotype
5. L/l	thrum homozygote lethality
6. Gm/gm	contributes to stylar length
7. Mpp/mpp	dominance (sporophytic nature) of male incompatibility phenotype
8. A/a	anther position

Loci 2 - 7 are all within the P linkage component but their relative positions are uncertain.

the pin is *gpa/gpa*. Long homostyles in *Primula* represent gPA and short homostyles Gpa.

A more recent study by Kurian and Richards (1997) confirmed previous studies and provided genetic evidence for further components of the *Primula S*-locus complex. A homostyle was identified which: possessed dimorphic pollen (50% pin / 50% thrum in size i.e. pollen size was gametophytically regulated); was compatible with self and pin mothers, but incompatible with thrum mothers; had style and stigma cells of an intermediate size between pin and thrum morphs. Through segregation analysis the authors were able to build substantially on previous work and provide a number of interesting insights: a) At least two loci control pollen size, one acting gametophytically and the other sporophytically. b) At least two loci control style length in an additive fashion within the *S*-locus complex and these control both style cell and stigma cell size, but not stigma morphology. c) Male and female incompatibilities are genetically distinct. d) A gene residing in the *S*-locus, controls the sporophytic nature of male incompatibility. e) The thrum *S*-allele appears to harbor a lethal gene, preventing the occurrence of its homozygotes.

This work combined with previous studies provides good evidence for the 8 genetic components of the locus shown in **Table 2**.

It thus appears that at least 8 genes (and possibly more) are present in the *Primula* heteromorphy supergene. These genes act in a coordinated manner to regulate both floral morphology and physiology and some are capable of superimposing sporophytic control on otherwise gametophytic aspects of this breeding system. Whilst knowledge of the composition of supergene complex has been gradually improving, the identity of the genes at these sub-loci remains to be determined. The fact that these genes are genetically linked promises to substantially aid the task of their identification.

FLORAL DEVELOPMENT IN *PRIMULA*

The ABC model of floral development provides a simple representation of floral development to define the 4 whorls of floral organs and is considered to be universally applicable to flowering plants (Bowman 1997). Though this model almost certainly applies to floral morphogenesis in *Primula*, there are clear differences in development between *Primula* and the species for which this model was developed (*Arabidopsis thaliana* and *Antirrhinum majus*). These include not only the processes that lead to heteromorphy, but also the temporal sequence in which the whorls develop. In *Arabidopsis* and *Antirrhinum* floral whorl development proceeds in the order: sepals, petals, stamens, carpels, i.e. centripetally. In contrast, it was noted as early as 1844 (Ducharte 1844) that in *Primula* whorl development does not progress in a linear order and proceeds: sepals, stamens, carpels, petals. Compared with *Arabidopsis*, which like *Primula* possesses flowers with radial symmetry, the pattern of floral organ fusion in *Primula* is also quite different. In *Arabidopsis* no organ fusion is seen in the first 3 whorls, in contrast in extensive organ fusion is seen in *Primula* in all three: the sepals are fused for over half their length to produce a calyx which surrounds the flower, the petals are fused at the based to form an elongated corolla tube and the stamen filaments are fused to the inside of the corolla tube (**Fig. 2**). Interestingly in *Primula* the petals and stamen, which represent the second and third whorls, arise from the same primordia (Webster and Gilmartin 2003) a phenomenon that has also been described in *Pisum sativum* (Fernándiz *et al.* 1999). These differences dictate that the molecular characterization of *Primula* floral development at the level is likely to provide novel insights above and beyond the standard ABC model, nonetheless the most intriguing aspects of this system lie in the processes regulating the onset of heteromorphy.

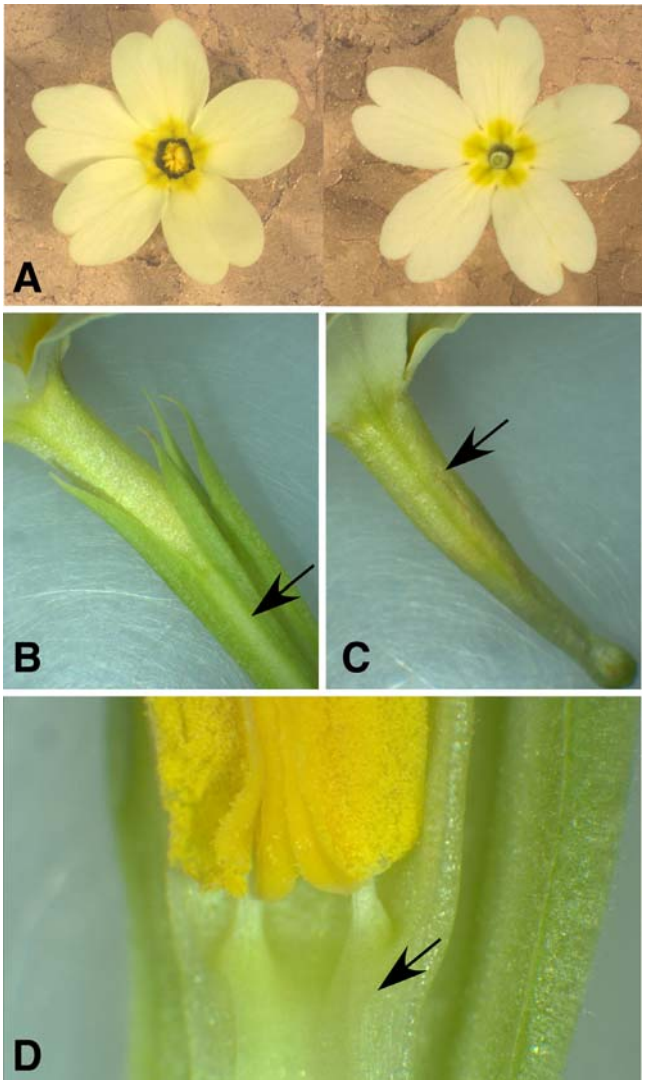

Fig. 2 *Primula* flower characteristics. (**A**) Radial symmetry in thrum and pin morphs. (**B**) Arrow indicates fusion of sepals to form a calyx. (**C**) Arrow indicates fusion of petals to form a corolla tube. (**D**) Arrow indicates the fusion of the anther filaments to the corolla tube.

A thorough characterization of the variations in development profiles between the *Primula* floral morphs has been recently achieved using scanning electron microscopy, and has provided a number of interesting insights. In the initial stages of development pin and thrum flowers are indistinguishable, their divergence begins only after the floral organs start to differentiate (Webster and Gilmartin 2003). As the floral forms diverge they do so in a way that suggests that the *A* and *G* components of the *S*-locus have different spatial and temporal functions. The first observable difference between morphs is seen in 5 mm buds (with a petal length of 2 mm), at which point the stigma is slightly elevated above the anthers in pin flowers while in thrum it is

slightly below. That style elongation is the first discernable step in morphological divergence suggests that the *G* component of the *S*-locus is the first to be implemented, leading to inhibition of style elongation by the dominant *G* allele in thrum flowers.

A difference in anther positioning (*A* function) is not discernable until 11 mm buds (with a 7 mm corolla), and hence the visible effects of *A* activity appear rather later than *G*. Anther positioning is mediated by differential physical growth of the corolla tube above and below the point of anther attachment (termed the upper and lower corolla tube respectively). Development of the upper corolla tube occurs prior to expansion of the lower corolla tube in both morphs, but elongation of the lower corolla tube initiates earlier in thrum than pin. In this case the *A* allele in the thrum morph plays a dominant role in promoting anther elevation by increasing growth of the lower corolla tube (to which the anther filament is fused). The difference in timing between *A* and *G* function, combined with the observation that the dominant alleles of *A* and *G* are growth suppressing and growth promoting respectively suggest that the two functions act through distinct mechanisms.

Studies of cell size and shape in the style and corollas also indicate that the mechanisms regulating style length and anther positioning differ. The difference in style length between morphs appears to predominantly result from differential cell elongation (Heslop-Harrison *et al.* 1981; Webster and Gilmartin 2006). Increased elevation of the anthers in thrum flowers, however, appears to result from differential cell division above and below the point of attachment to the corolla tube of pin and thrum flowers. These differences only become significant as the flowers approach maturity, suggesting that anther height in thrum flowers is a result of increased cell division below the point of anther attachment (Webster and Gilmartin 2006).

In the course of these studies a novel polymorphism between pin and thrum flowers was uncovered, the thrum corolla tube mouth in thrum flowers is wider than the pin. This increase in diameter is caused by an increase in width of the cells of the upper corolla tube in thrum as compared to pin flowers. Developmentally, the corolla tube below the anthers of the thrum elongates early in development (7 mm) while the pin corolla does not elongate until later (11 mm) in development (Webster and Gilmartin 2006). This timing and the fact that corolla cells are involved in anther elevation, led to the proposal that this difference is a direct consequence of the action of *A* component of the *S*-locus.

Interestingly a number of horticultural varieties of *Primula* possess floral mutations not related to heteromorphy, but that are known to be either completely or partially, linked to the *Primula S*-locus (see Webster and Gilmartin 2003, 2006). These include *Hose in Hose* (petals develop in place of sepals), *sepaloid* (phenotype varies from 2 or 3 whorls of sepals around a carpel to 4 whorls of sepals), *stamenoid carpels* (sepals replaced by petals and ovary converted to stamen enclosing naked ovules), and the floral colors *magenta* and *maroon* (see Webster and Gilmartin 2003, 2006). The molecular genetic basis of the floral mutations in these varieties is unknown, but their linkage to the *S*-locus suggests that molecular analysis of the *S*-locus region is likely to lead to the identification of a number of genes involved in floral development above and beyond those involved directly in heteromorphy.

MOLECULAR WORK ON THE HSI *S*-LOCUS

Currently there are remarkably few molecular analyses of HSI systems to be found in the literature. Such studies have begun in *Turnera* sp. (Anthanasiou and Shore 1997; Khosravi *et al*. 2006), buckwheat (*Fagopyrum homotropicum*) (Aii *et al*. 1999) and, more recently, *Primula* (Manfield *et al*. 2005; McCubbin *et al*. 2006). HSI systems provide an excellent opportunity to identify genes controlling a number of important reproductive traits. Traits such as pollen size and number and style length are subtle relative to organ identity and are less tractable to mutant screening. As the traits of HSI are genetically linked within the *S*-locus, physical mapping approaches promise to be an effective way to speed their identification. The first step to physical mapping of the *Primula S*-locus is to generate molecular markers. There are a wide variety of approaches through which this might be achieved, the most significant difference between them being whether they are based on genomic DNA or cDNA.

Using a genomic DNA based strategy, random amplification of polymorphic DNA (RAPD)-PCR, a marker linked to the dominant thrum *S* allele has been identified (Manfield *et al*. 2005). Characterization of this DNA fragment to generate a sequence characterized amplified region (SCAR) marker (Genbank Accession # AY854262) provided a molecular probe that was used to isolate marker an 8.8 kb clone representing genomic DNA from the thrum allele of the *S*-locus. This clone has been fully characterized, and though no genes were found in this region, analysis of the DNA sequence revealed some interesting features. The DNA sequence of this region was found to be highly repetitive and had a structure reminiscent of that found in *S*-loci of species with homomorphic self-incompatibility systems. A significant number of retrotransposon-like elements were also identified. The common feature between *S*-loci of all systems is the need to maintain genetic linkage between the multiple genes they require to function. The repetitive sequence structure most likely reflects the nature of the HSI *S*-locus as a linkage group that has accumulated structural elements that help to suppress recombination between alleles.

cDNA based approaches for identifying molecular markers have the advantage that they encode genes, but the disadvantage of that differential gene expression can dramatically complicate analyses. Using the approach of subtractive suppressive hybridization, McCubbin and co-workers (2006) identified 11 classes of cDNA which exhibited differential expression between at least one floral tissue of developing of pin and thrum flowers. None of these genes were linked to the *S*-locus and they were interpreted as being components of pathways downstream of the developmental switches located at the *S*-locus. A number of these genes were found to have homology to gene families known to be involved in developmental processes, including rapid alkalinization factors and *CHX* ion transporters which have been implicated in cell expansion, and *DExH* box RNA helicases and *SKS* multicopper oxidases which have been implicated in regulation cell size. It seems likely that at least some of these genes are involved processes regulating floral morphology however further characterization is necessary before involvement in a particular developmental process can be assigned.

CONCLUSIONS

Though our overall knowledge of how the HSI system in *Primula* is established is still scant, recent studies using both electron microscopy and molecular biology have provided novel insights as well substantiating previous hypotheses concerning the genetic composition of the *S*-locus. Most these studies are generating tools that will facilitate the identification of all the components of the *Primula S*-locus in the near future. In addition to the published studies, we are aware of the generation of as yet two unpublished genomic bacterial artificial chromosome libraries of *Primula vulgaris* that have been generated, which will greatly expedite the task of chromosome walking and gene identification in this highly repetitive linkage group.

Aside from the academic interests that will be fulfilled by gaining a full understanding of the molecular regulation of HSI, there are important potential applications to agriculture and horticulture. The manipulation of floral form is an obvious goal in creating novel horticultural products. From an agricultural perspective, one of the biggest concerns about the current adoption of genetically modified crops is transgene escape - both to wild relatives of crop species and

to non-transgenic cultivars. Identification of the suite of genes that regulate the co-adapted reproductive traits found in HSI, promise to provide us with a set of tools through which we might manipulate the breeding systems of at least eudicot species. These tools would facilitate the concerted engineering of male and female traits with the goal of generating plants with improved breeding barriers to wild relatives, novel breeding barriers to non-transgenic cultivars and yet which remain self-fertile - effectively to make them into distinct species. The author of "The Origin of Species" would surely be amused by the irony of employing one of his favorite model systems to artificially originate species to mitigate a serious problem in the modern world.

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