Chapter 1

New Insights on Heterostyly: Comparative Biology, Ecology and Genetics

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Abstract Here, we review recent progress on the evolutionary history, functional ecology, genetics and molecular biology of heterostyly using a variety of taxa to illustrate advances in understanding. Distyly and tristyly represent remarkable examples of convergent evolution and are represented in at least 28 flowering plant families. The floral polymorphisms promote disassortative mating and are maintained in populations by negative frequency-dependent selection. Comparative analyses using phylogenies and character reconstruction demonstrate multiple independent origins of heterostyly and the pathways of evolution in several groups. Field studies of pollen transport support the Darwinian hypothesis that the reciprocal style–stamen polymorphism functions to increase the proficiency of animal-mediated cross-pollination. Although the patterns of inheritance of the style morphs are well established in diverse taxa, the identity, number and organization of genes controlling the heterostylous syndrome are unknown, despite recent progress. In future, it will be particularly important to establish the contribution of 'supergenes' vs. regulatory loci that cause morph-limited expression of genes.

Abbreviations

2D PAGE	Two-dimensional polyacrylamide gel electrophoresis
GPA locus	Gynoecium, Pollen size, Anther height
IEF	Isoelectric focussing
L-morph	Long-styled morph

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M-morph	Mid-styled morph
mRNA	messenger RNA
P^*	Abnormal pin morph
PvSLL1	cDNA from *Primula* linked to the *S-locus*; encodes a small putative transmembrane protein of unknown function
PvSLL2	cDNA from *Primula* closely linked to the *S-locus*; has homology to the *CONSTANS-LIKE* gene
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SI	Self-incompatibility
S-locus	Self-incompatibility locus
S-morph	Short-styled morph

1.1 Introduction

The sexual organs of most flowering plants exhibit a small degree of continuous variation resulting from quantitative inheritance and environmental influences. A strikingly different pattern of variation is evident in populations of some species, where hermaphroditic individuals fall into two or three morphologically distinct mating groups, which differ in style length, anther height and a suite of ancillary pollen and stigma polymorphisms (Darwin 1877; Vuilleumier 1967; Ganders 1979; Barrett 1992). Populations with this type of polymorphic sexual variation are distylous or tristylous, respectively, and the general condition is referred to as heterostyly.

The defining feature of heterostylous populations is a reciprocal arrangement of sex-organ heights in the floral morphs (Fig. 1.1), also known as reciprocal herkogamy (Webb and Lloyd 1986). By convention, the morphs are referred to as long- and short-styled (hereafter L- and S-morphs) in distylous populations, and long-, mid- and short-styled (hereafter L-, M- and S-morphs) in tristylous populations. In most heterostylous species, reciprocal herkogamy is associated with a hetermorphic self-incompatibility (SI) system that limits or prevents self- and intra-morph mating. Therefore, compatible pollinations occur only between anthers and stigmas of equivalent height, termed 'legitimate pollinations' (Fig. 1.1; Darwin 1877). Understanding the evolution, function and genetic basis of heterostyly has attracted considerable attention since Darwin's classic book on polymorphic sexual systems in plants (Darwin 1877). The sustained fascination with heterostyly occurs because the sexual polymorphisms are a remarkable example of convergent evolution. In addition, they represent one of the classic research paradigms for the study of evolution and adaptation in plants (Barrett 1992).

Darwin (1877) provided the earliest adaptive explanation for the function of heterostyly. He proposed that the reciprocal placement of anthers and stigmas was a mechanism to promote pollinator-mediated cross-pollination between floral morphs.

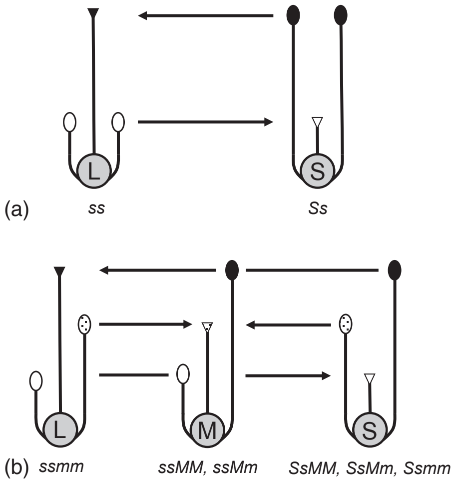

Fig. 1.1 The heterostylous floral polymorphisms: (**a**) distyly and (**b**) tristyly. L, M and S refer to the long-, mid- and short-styled morphs, respectively. The arrows indicate cross-pollinations between anthers and stigmas of equivalent height. In the majority of heterostylous species these are the only compatible pollinations. Genotypes for the floral morphs with the most common patterns of inheritance are indicated (see Sect. 1.4.1. for further details)

According to Darwin's hypothesis, pollinators visiting heterostylous flowers pick up pollen on different parts of their bodies during nectar feeding, and cross-pollen transfer between floral morphs is promoted by this segregated pollen deposition. Several lines of evidence support Darwin's cross-pollination hypothesis (Kohn and Barrett 1992; Lloyd and Webb 1992b) and heterostyly is generally described as an 'outcrossing mechanism'. However, this interpretation is insufficient for two reasons. First, self-incompatibility (SI) in heterostylous populations guarantees outcrossing, and, second, by preventing intra-morph mating heteromorphic SI restricts mating opportunities with one half (distyly), or one third (tristyly), of the plants in a population. A more complete interpretation of the adaptive significance of heterostyly recognizes different functional roles for the morphological and physiological components of the polymorphism in promoting fitness through male and female function, respectively.

Reciprocal herkogamy, as Darwin (1877) proposed, functions to promote proficient cross-pollination by reducing male gamete wastage on incompatible stigmas and increasing fitness through male function (Lloyd and Webb, 1992a, b). In contrast, SI safeguards against self-fertilization and inbreeding depression, thereby promoting the maternal component of fitness. Recognition of these different paternal and maternal functions resolves the apparent redundancy of two mechanisms with essentially the same role. The evolution of heterostyly reduces the conflict that can occur in sexually monomorphic animal-pollinated species—achieving efficient cross-pollination but simultaneously avoiding self-interference between female and male sexual organs (Barrett 2002).

Studies of heterostyly have largely concentrated on a few well-characterized taxa (e.g. *Primula, Linum, Lythrum*) originally studied by Darwin (1877). Of these, *Primula* has attracted most attention and is often represented in the literature as the model system for heterostyly (Mast and Conti 2006; see also Chap. 14, this volume). However, heterostyly is now reported from at least 28 angiosperm families and the polymorphism has evolved on numerous occasions. In addition, other stylar polymorphisms have also been recognized with their own distinctive features [e.g. stigma-height dimorphism (Baker et al. 2000a, b; Barrett et al. 2000a); enantiostyly (Barrett 2002; Jesson and Barrett 2003); flexistyly (Li et al. 2001; Renner 2001; Sun et al. 2007); inversostyly (Pauw 2005)] raising new questions about their evolution, function and relation to heterostyly.

Early research on heterostyly largely focused on genetical aspects of the polymorphism. Indeed, fundamental concepts in Mendelian and population genetics, including patterns of inheritance, linkage, supergenes, epistasis and polymorphic equilibria, were initially studied in *Primula* and *Lythrum* by leading geneticists, including W. Bateson, R.A. Fisher, J.B.S. Haldane, A. Ernst. A.B. Stout, K. Mather and D. Lewis. Today, a much broader range of questions are being addressed in heterostylous species employing diverse approaches. The objective of this chapter is to review recent advances on the study of heterostyly by examining progress made since the last general treatment (Barrett 1992). We review work on the comparative biology, ecology and genetics of heterostyly and conclude by briefly outlining future research for solving outstanding problems remaining in the study of heterostylous plants. A goal of our review is to demonstrate that although heterostyly is probably the most well studied plant sexual polymorphism, there still remain many unanswered questions that require future investigation.

1.2 Comparative Biology and Evolutionary History of Heterostyly

Heterostyly has a scattered distribution among at least 28 angiosperm families with new heterostylous taxa continuely reported [e.g. distyly in *Aliciella* formerly *Gilia* (Polemoniaceae; Tommerup 2001); *Salvia* (Lamiaceae; Barrett et al. 2000b); and *Tylosema* (Caesalpinioideae; Hartley et al. 2002); tristyly in *Hugonia* (Linaceae; Thompson et al. 1996)]. Lloyd and Webb (1992a) surveyed the character states of 25 families with heterostylous species to identify why the polymorphism may have evolved in some families and not others. Their analysis indicated that there are constraints on the types of flowers in which reciprocal herkogamy is likely to evolve. Heterostylous flowers are usually actinomorphic with a simple open corolla and a floral tube with nectar concealed at the base. These flowers are described as stereomorphic or ‘depth-probed’ (Lloyd and Webb 1992a), with sexual organs contacted by long-tongued pollinators in succession during nectar feeding. Heterostyly is rarely associated with strongly zygomorphic flowers, probably because in such groups effective cross-pollen transfer is achieved through pollinator positioning.

Families in which flowers possess numerous stamens, free carpels, open-dished shaped corollas and exposed nectar usually lack the precision in pollen transfer required for the evolution of reciprocal herkogamy. However, exceptions to these patterns exist in heterostylous taxa [e.g. zygomorphy in *Salvia* (Barrett et al. 2000b); open dish- or bowl-shaped flowers in *Fagopyrum* (Bjorkman 1995) and *Turnera* (Rama Swamy and Bahadur 1984); numerous stamens and nectar-less flowers in *Hypericum* (Ornduff 1975)], raising the question of how reciprocal herkogamy evolved and is maintained in these taxa.

1.2.1 Phylogeny Reconstruction and Character Evolution

The advent of molecular systematics, phylogeny reconstruction and character mapping has led to interest in the evolutionary history of SI. See Chap. 4 for consideration of the evolutionary history of homomorphic SI. Here, we consider studies of heterostylous taxa, which include Pontederiaceae (Kohn et al. 1996), *Amsinckia* (Schoen et al. 1997), *Houstonia* (Church 2003); *Primula* (Mast et al. 2004), *Narcissus* (Graham and Barrett 2004); *Linum* (Armbruster et al. 2006), *Turnera* (Truyens et al. 2005) and Lythraceae (Morris 2007). Phylogenetic analyses of these groups have been conducted to address questions concerning the origin and evolutionary history of heterostyly and related sexual systems, and these have included the following: (1) Has heterostyly evolved more than once in a particular lineage? (2) What are the ancestral states and intermediate stages involved in the evolution of heterostyly? (3) What is the order of establishment of morphological and physiological traits in the heterostyous syndrome? (4) What are the evolutionary relationships between heterostyly and related stylar conditions, including homostyly? Part of this work has been motivated by efforts to distinguish between the predictions of competing theoretical models on the evolution of heterostyly (Charlesworth and Charlesworth 1979a; Lloyd and Webb 1992a, b).

Lloyd and Webb (1992a) estimated that heterostyly originated on at least 23 separate occasions, based on its distribution among 19 orders of flowering plants, but conceded that many more origins may be involved if multiple origins have occurred within heterostylous taxa. Although several studies have assumed that heterostyly is the basal condition in lineages (Schoen et al. 1997; Truyens et al. 2005), other evidence points to multiple origins of heterostyly within some genera (Graham and Barrett 2004). Inferences on the number of origins of heterostyly within particular groups may be particularly sensitive to taxon sampling and the weighting schemes employed for the gain and loss of heterostyly [e.g. Pontederiaceae (Kohn et al. 1996); *Linum* (Armbruster et al. 2006); *Primula* (Mast et al. 2006)]. In large geographically widespread families with numerous heterostylous species, such as Rubiaceae and Oxalidaceae, the polymorphism may have had multiple origins. If this turns out to be the case, it will be interesting to investigate the details of each transition, including their ecological and developmental basis and if the patterns of inheritance are similar.

Determining the ancestral states of heterostylous lineages is of importance for distinguishing models of the evolution of heterostyly. Lloyd and Webb (1992a, b) proposed that the immediate ancestors of distylous species were monomorphic for style length and possessed approach herkogamy, with long styles and stamens positioned below the stigma. In contrast, in the model of Charlesworth and Charlesworth (1979a), the ancestral condition involves a population with long styles and long-level stamens. Both models assume that distyly evolves via an intermediate stage of stigma-height dimorphism with short-styled variants invading long-styled monomorphic populations.

Narcissus offers an opportunity to evaluate these models because the genus contains species with stylar monomorphism, stigma-height dimorphism and distyly (Barrett et al. 1996). Reconstruction of the evolutionary history of stylar conditions provides evidence to support the stages envisioned in the Lloyd and Webb model (Graham and Barrett 2004). As shown in Fig. 1.2, the immediate ancestors of

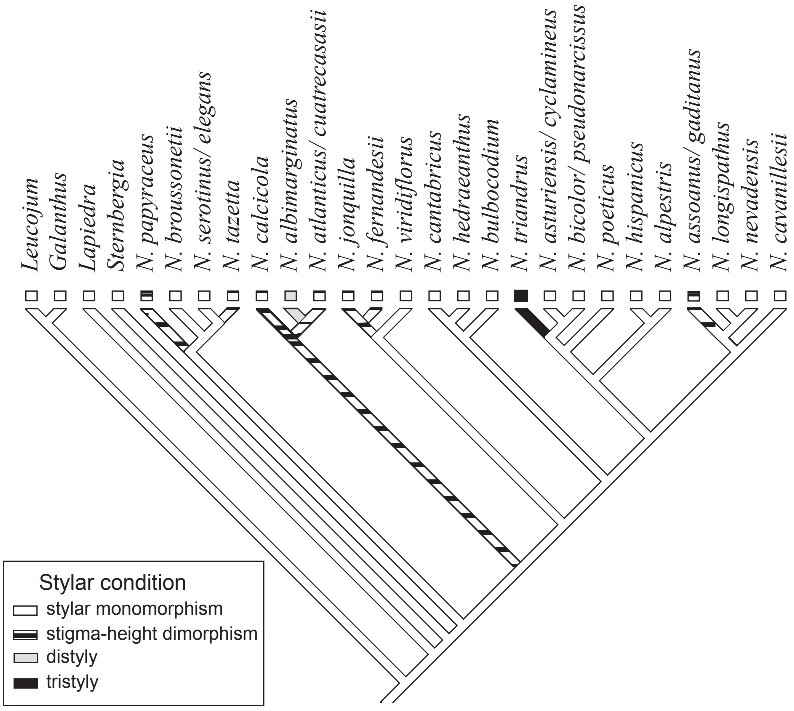

Fig. 1.2 Reconstruction of the evolutionary history of stylar polymorphisms in *Narcissus* based on parsimony analysis of combined *ndh*F and *trn*L–*trn*F DNA sequences. Some taxa with identical stylar conditions are reduced to single termini. Six origins of stylar polymorphism are inferred, with independent origins of distyly and tristyly and multiple origins of stigma-height dimorphism. The single distylous species, *N. albimarginatus*, is descended from ancestors with stigma-height dimorphism supporting theoretical models that propose stigma-height dimorphism as an intermediate stage in the evolution of distyly, see Sect. 1.2.1 for further details. After Graham and Barrett (2004), figure published with permission from *New Phytologist*

the sole distylous member of the genus (*N. albimarginatus*) possess stigma-height dimorphism, and the basal condition in the genus is stylar monomorphism and approach herkogamy. The rarity of stigma-height dimorphism in flowering plants (Barrett et al. 2000a) limits opportunities to examine these models more generally. However, reconstruction of the evolutionary history of stylar conditions in Boraginaceae would be of value because this family includes genera (e.g. *Lithodora*) with both distyly and stigma-height dimorphism.

Models of the evolution of heterostyly also differ in the order in which the morphological and physiological components of heterostyly are established. In the Charlesworth and Charlesworth (1979a) model, the origin of diallelic SI is a prerequisite for the subsequent evolution of reciprocal herkogamy, whereas in Lloyd and Webb's (1992a, b) model, the style–stamen polymorphism establishes first and diallelic incompatibility may, or may not, follow depending on the importance of selection against the genetic costs of self-fertilization. It has been difficult to obtain comparative evidence to support either sequence, in part, because SI systems in heterostylous groups commonly exhibit considerable variation in expression. This ranges from self-compatibility (SC), through cryptic self-incompatibility, to differences in the strength of incompatibility among the floral morphs, to rigid SI in all morphs (reviewed in Barrett and Cruzan 1994). This variation complicates character reconstructions, as does the problem of determining whether the self-compatible status of some heterostylous species represents an ancestral or derived condition. These issues were encountered in the only explicit attempt to determine the order of establishment of reciprocal herkogamy and incompatibility, in tristylous Pontederiaceae (Kohn et al. 1996). The isolated case of distyly in self-compatible *Salvia brandegeei* of the Lamiaceae is noteworthy because there are no reliable reports of SI in this family (Barrett et al. 2000b). This observation supports the Lloyd and Webb view that reciprocal herkogamy can establish independently of diallelic incompatibility.

The status of stylar monomorphism is contentious in heterostylous groups as it could represent either an ancestral or derived condition. In *Primula*, 'secondary homostyly' through recombination of the linkage group controlling distyly is well known (Charlesworth and Charlesworth 1979b; and see Sect. 1.4.2.). However, several authors have interpreted monomorphism in some *Primula* species as representing the ancestral condition in the genus prior to the origin of distyly (Ernst 1955; Al Wadi and Richards 1993). Recent phylogenetic analyses of *Primula* cast doubt on this interpretation, suggesting instead that species with stylar monomorphism are more likely to be derived from distylous ancestors (Mast et al. 2006). Among the derived monomorphic taxa are members of *Dodecatheon*, a group of buzz-pollinated species (Mast et al. 2004, 2006). This indicates an intriguing shift in pollination system from distyly, in which pollen dispersal is based on nectar feeding and the geometry of pollinator contacts, to a strikingly different strategy based on nectarless flowers and metered pollen dispensing from anthers with poridical dehiscence. Mast et al. (2004) detail the character-state changes associated with this transition, but nothing is known about the ecological mechanisms responsible for this transition in pollination system.

Self-pollinating homostyles commonly occur in heterostylous groups. In *Eichhornia* (Kohn et al. 1996), *Amsinckia* (Schoen et al. 1997) and *Turnera* (Truyens et al. 2005), phylogenetic reconstructions have provided good evidence that homostyles are derived from heterostylous ancestors. The shift to predominant selfing has occurred on multiple occasions within each genus, probably as a response to selection for reproductive assurance under conditions of unreliable pollinator service. Although the acquisition of homostyly is commonly associated with the evolution of small flowers and the loss of floral traits promoting outcrossing, as in *Amsinckia* and *Eichhornia*, this is not the only evolutionary outcome. In *Turnera*, some homostylous populations have large flowers, display a moderate degree of herkogamy and exhibit mixed mating systems (Belaoussoff and Shore 1995). Homostylous populations retain residual diallelic incompatibility reactions, suggesting that they originated as long-homostyled recombinants with stigmas and anthers at equivalent heights (Tamari et al. 2001 and see Sect. 4.2.). Selection on quantitative genetic variation governing the degree of herkogamy has led to increased outcrossing rates (Barrett and Shore 1987; Shore and Barrett 1990), illustrating the potential evolutionary lability of mating patterns in some homostylous populations. The transition from heterostyly to homostyly is the most frequent evolutionary change in floral biology and mating system that has occured among heterostylous groups. Future work on the phylogeny of heterostylous taxa is critical for providing a comparative context for investigating the ecological, genetic and developmental basis of this shift from outcrossing to selfing.

1.3 Function and Reproductive Ecology of Heterostyly

Heterostyly is a conspicuous floral polymorphism that can be readily identified under field conditions. Consequently, there is growing interest in using the polymorphism to address diverse questions in functional ecology, ecological genetics and conservation biology. Here we review recent studies on these topics.

1.3.1 Function of Heterostyly

The occurrence of pollen-size heteromorphism in heterostylous species enables measurement of disassortative (inter-morph) pollen transfer based on the analysis of stigmatic pollen loads. Data obtained from ‘pollen flow’ studies can be used to evaluate Darwin’s cross-promotion hypothesis for the adaptive significance of heterostyly. Ganders (1979) and Lloyd and Webb (1992b) summarized experimental evidence supporting the Darwinian hypothesis, based largely on studies of bee-pollinated species from temperate ecosystems. Since then additional studies, particularly on tropical members of Rubiaceae with diverse pollinators (e.g. bird, butterfly), have provided further support that reciprocal herkogamy promotes inter-morph pollen transfer (Stone 1995; Ree 1997; Pailler et al. 2002; Lau and Bosque 2003; Massinga et al. 2005; Hernandez and Ornelas 2007). In common with earlier work, marked asymmetries between the floral morphs in total pollen transfer and the capture of compatible pollen were evident. Stigmas of the L-morph capture more total pollen, but the proportion of compatible pollen on stigmas of the S-morph is generally higher.

A survey of pollen transfer efficiency in distylous species found that in 13 of 17 studies pollen grains of the S-morph were more likely to be transferred to compatible stigmas than pollen grains of the L-morph (Stone and Thomson 1994). This probably occurs because long-level organs are more accessible to pollinators. However, the direction of asymmetry reported can depend on the particular estimates of efficiency used, whether morph ratios and pollen production are taken into account, and if flowers are left intact or are emasculated (Ganders 1979; Stone 1995; Pailler et al. 2002). The cause of pollen transfer asymmetries lie in the interaction between floral morphology and the entry and exit paths of pollinators during nectar feeding (Ganders 1979; Lloyd and Webb 1992b; Stone 1995). Although the specific mechanical details need to be determined for individual cases, the overall patterns appear to be quite general, given the consistent results reported across diverse floral morphologies and pollinator groups.

Darwin's cross-promotion hypothesis has recently been examined in *Narcissus* species with stylar polymorphisms. This group presents a particular challenge because the floral morphs produce pollen of uniform size and it is not possible to determine directly the source of pollen deposited on stigmas. Instead, field manipulations have been employed to investigate pollen transfer. By altering the morph composition of local patches of *N. assoanus*, a species with stigma-height dimorphism, Cesaro and Thompson (2004) inferred higher rates of inter-than intra-morph pollination based on measurements of female fertility. However, this effect was evident only in pollen transfer from the L-morph to the S-morph, not in the reverse direction. Despite asymmetrical disassortative pollination, the levels reported were sufficient to maintain style-length dimorphism, and also satisfy theoretical conditions necessary for the evolution of the polymorphism under pollen-limited conditions (Lloyd and Webb 1992b). Other manipulative field experiments with *N. assoanus*, demonstrating negative frequency-dependent reproductive success also provide evidence for inter-morph pollen transfer in maintaining stylar dimorphism (Thompson et al. 2003).

In most heterostylous populations, regardless of the patterns of pollen transfer, diallelic incompatibility governs realized mating patterns, resulting in symmetrical disassortative mating and equal morph ratios, at least in equilibrium populations. However in *Narcissus* species, late-acting ovarian SI limits selfing but permits both intra- and inter-morph mating (Sage et al. 1999; see also Chap. 4, this volume). Therefore, differences in the floral morphology of the morphs influence mating patterns more strongly than those in typical heterostylous species. Specifically, opportunities for intra-morph mating in the L-morph occur because this morph has anthers positioned close to stigmas of long styles. Theoretical models indicate that asymmetrical mating resulting from incomplete sex-organ reciprocity could explain the prevalence of L-morph biased ratios in *Narcissus* species with stylar polymorphisms (Barrett et al. 1996; Baker et al. 2000b; Hodgins and Barrett 2006). Recent investigations provide empirical support for these models by showing a functional link between floral morphology, asymmetrical mating and biased morph ratios.

In tristylous *N. triandrus*, the L-morph possesses long-level rather than mid-level stamens (Barrett and Hodgins 2006). Populations exhibit strongly L-morph biased ratios throughout the range and pollen transfer models implicate assortative mating in the L-morph as the cause of biased ratios (Barrett et al. 2004; Hodgins and Barrett 2006). Paternity analysis of offspring from open-pollinated progenies was recently used to estimate rates of intra- and inter-morph mating (Hodgins and Barrett 2008). Patterns of outcrossed siring success indicated levels of assortative mating in the L-morph sufficient to cause L-morph bias. However, despite this result, overall levels of inter-morph transfer exceeded intra-morph transfer, supporting predictions of the Darwinian hypothesis that reciprocity of sex-organ position promotes pollinator-mediated cross-pollination.

1.3.2 Floral Morph Ratios and Reproductive Success

Reproductive success in species with stylar polymorphisms should be sensitive to plant density and the morph composition of local neighbourhoods. To investigate the spatial ecology of mating, Stehlik et al. (2006) mapped the location of floral morphs in *N. assoanus* populations and determined their female fertility. They found that pollen transfer and mating was context dependent, with the floral morphs responding differently to the density and morph identity of plants in local patches. Studies of *Narcissus* demonstrate that biased style morph ratios can be an equilibrium expectation if the strength of negative frequency-dependent selection varies among the morphs because of asymmetrical mating. However, because most heterostylous species possess heteromorphic incompatibility, biased morph ratios usually result from non-equilibrium conditions associated with the ecology of populations.

Biased morph ratios are particularly common in species with prolific clonal propagation (Barrett and Forno 1982; Castro et al. 2007). The signature of founder effects is often preserved over long periods, and progress towards morph-frequency equilibrium depends on the regularity of sexual reproduction and the demographic characteristics of populations (Eckert and Barrett 1995). In species with strong heteromorphic incompatibility, extensive clonal spread can interfere with sexual reproduction, resulting in a deficit of inter-morph cross-pollination and reduced seed set (Thompson et al. 1998; Ishihama et al. 2003; Brys et al. 2007; see also Chap. 3, this volume, with respect to the evolutionary implications of clonal propagation on the evolutionary dynamics of homomorphic SI). Species of *Nymphoides*, a genus of clonal aquatics, commonly exhibit biased morph ratios or stylar monomorphism, resulting in pollen limitation of fruit set and restricted sexual recruitment (Ornduff 1966; Shibayama and Kadono 2003; Wang et al. 2005a). By manipulating the frequency of flowers of the L- and S-morphs in an experimental population

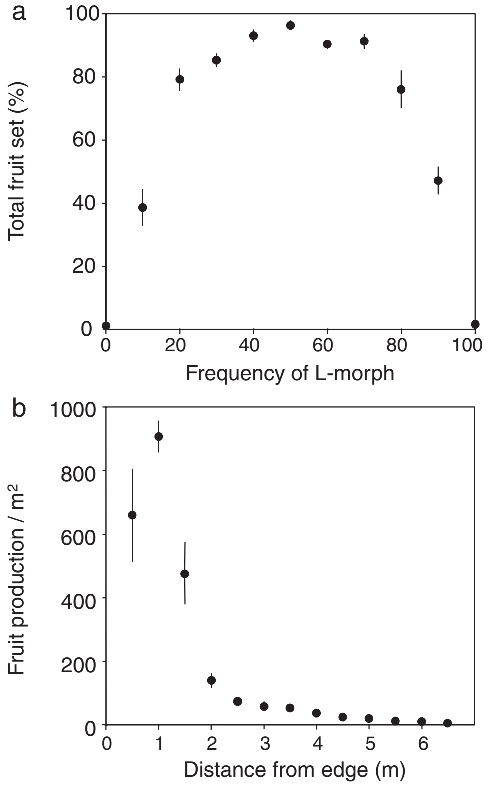

Fig. 1.3 Frequency-dependent (**a**) and proximity-dependent (**b**) reproductive success in distylous *Nymphoides peltata* (Menyanthaceae), an aquatic species with prolific clonal growth and strong dimorphic incompatibility. In (**a**) the ratio of long- and short-styled flowers in an experimental population influence total fruit set and biased ratios reduce maternal fertility because of a deficit of compatible pollen. In (**b**) fruiting density per square meter declines steeply with distance from the nearest compatible mating partner (S-morph) in a large clonal patch of the L-morph. After Wang et al. (2005a), figure published with permission from *New Phytologist*

of *Nymphoides peltata*, Wang et al. (2005a) demonstrated the frequency-dependent nature of reproductive success in distylous populations and also how extensive clone size can limit fruit set (Fig. 1.3).

Founder events, population bottlenecks and genetic drift can have significant ecological and evolutionary consequences, particularly through the loss of morphs from heterostylous populations (Barrett et al. 1989; Eckert and Barrett 1992). Also, small populations are often less attractive to pollinators, resulting in pollinator limitation of seed set in self-incompatible species (Ågren 1996). The growth of conservation biology has led to interest in the extent to which small population size resulting from habitat fragmentation influences the reproductive success of heterostylous populations. This work has largely focused on declining populations of distylous species of *Primula* occurring in human dominated landscapes of Japan [e.g. *P. sieboldii*

(Washitani et al. 2005)] and Europe [e.g. *P. vulgaris* (Jacquemyn et al. 2003; Kéry et al. 2003; Brys et al. 2004)]. These studies demonstrate that in small fragmented populations, the combined effects of pollinator losses and the scarcity of compatible morphs can limit reproductive success.

1.4 Inheritance of Heterostyly and the Supergene Model

The genetics of heterostyly was last reviewed by Lewis and Jones (1992); here we briefly outline salient work on the inheritance of distyly and tristyly to provide a background for our review of molecular investigations aimed at identifying the 'heterostyly genes'. We follow the convention of referring to the locus (or loci) determining distyly and tristyly as the *S*-locus, and the *S*- and *M*-loci, respectively. However, it is important to recognize that the '*S*-locus' in species with heteromorphic self-incompatibility is unlikely to be homologous with the *S*-locus of species with homomorphic SI (Gibbs 1986), described elsewhere in this volume; see also Chap. 14. The heterostyly loci are diallelic and appear to determine the entire syndrome of morphological and physiological traits that distinguish the floral morphs. A few cases are known in which self-incompatible heterostylous species do not possess diallelic incompatibility and instead may possess a multi-allelic incompatibility system (e.g. *Anchusa*, *Narcissus*, reviewed in Barrett and Cruzan 1994). In these genera, SI is not associated with the morphological traits that distinguish the morphs. However, the details of the type of SI involved (gametophytic vs. sporophytic) and its genetic basis have not been established, although at least two loci appear to be responsible in *A. officinalis* (Schou and Philipp 1984).

1.4.1 Inheritance of Distyly and Tristyly

Prior to the rediscovery of Mendelian inheritance, Darwin (1877) had already carried out the crosses necessary to partly, or wholly, deduce the patterns of inheritance of heterostyly in several taxa. Subsequently, Bateson and Gregory (1905) elucidated the inheritance of distyly in *Primula sinensis*, demonstrating the segregation of a dominant (S) and a recessive (s) allele governing the L-morph (ss) and S-morph (Ss) at what was considered to be a single locus. Today, the inheritance of distyly has been investigated in species from ∼10 heterostylous families (Lewis and Jones 1992). All species exhibit the *Primula* inheritance pattern, although the dominance relations are reversed in *Armeria*, possibly *Limonium* (Baker 1966), and in *Hypericum* (Ornduff 1979). Lloyd and Webb (1992a) provide a compelling evolutionary explanation for the similar pattern of inheritance of distyly among heterostylous families.

The genetics of tristyly has also had a long history of investigation through studies of Lythraceae and Oxalidaceae (Barlow 1923; Fisher and Mather 1943;

Weller 1976; Eckert and Barrett 1993). Inheritance involves two diallelic loci with the *S*-locus epistatic to the *M*-locus. Plants carrying at least one copy of the *S*-allele are of the S-morph, and those that are homozygous recessive at the *S*-locus are either of the M-morph (*ssMM* and *ssMm*) or the L-morph (*ssmm*). The two loci segregate independently in *Decodon verticillatus* (Eckert and Barrett 1993) and *Lythrum salicaria*, which exhibits tetrasomic inheritance (Fisher 1941; Fisher and Mather 1943). In contrast, the loci are linked in most *Oxalis* species (Fisher and Martin 1948; Fyfe 1950; Weller 1976), and in *Eichhornia paniculata* of Pontederiaceae (Barrett unpublished data), but not in *Pontederia cordata* (Gettys and Wofford, 2008). More complex patterns of inheritance, including recessive epistasis and an additional locus, are reported from other *Oxalis* species (Fyfe 1956; Bennett et al. 1986; Trognitz and Hermann 2001). As yet there is no obvious explanation as to why similar patterns of inheritance involving two loci and epistasis have originated independently in three unrelated tristylous families.

1.4.2 Supergene Model

Largely as a result of work by Alfred Ernst on *Primula*, details of the genetic architecture of distyly emerged from studies of the inheritance of novel floral phenotypes obtained from intra- and inter-specific crosses (Ernst 1955). Ernst demonstrated that self-compatible long homostyes were inherited as if determined by additional alleles at the *S*-locus, and proposed that two (Ernst 1928), and later showed that three, tightly linked loci were responsible for the inheritance of distyly (Ernst 1955). This early work essentially established the ‘supergene model’ for *Primula*. A supergene is a series of two or more tightly linked genes that function together to produce adaptive characteristics (Lewis and Jones 1992). Later, Dowrick (1956) extended the supergene model for *Primula* by revising the number and order of loci. The inference that a series of tightly linked diallelic loci determines distyly was made, in part, because the rate of appearance of novel floral phenotypes by recombination was greater than could be accounted for by mutation to new alleles.

Today, the number of loci comprising the supergene in *Primula* is usually assumed to be three linked diallelic loci (Charlesworth and Charlesworth 1979a; Lewis and Jones 1992; but see below and Chap. 14, this volume). The *G* locus determines characteristics of the gynoecium, including style length and its incompatibility response, *P* determines pollen size and its incompatibility response and *A* determines anther height. The three dominant alleles at each locus are linked in coupling and comprise the apparent ‘*S*-allele’ (*GPA*) with the three recessive alleles comprising the ‘*s*-allele’ (*gpa*). Lewis and Jones (1992) re-analyzed the data of Ernst and determined the order and map distance for the three loci. However, additional loci have been proposed to account for other features of dimorphism. For example, Dowrick (1956) argued that there are an additional four loci for the incompatibility specificities of pollen and styles, area of style transmitting tissue and stigmatic papillae length, thus increasing the count to seven loci with all alleles linked in coupling.

This inference was not based on the analysis of recombinants, which in some cases would be very hard to detect. Alternatively, it has been suggested that some of the ancillary dimorphisms (e.g. papillae length) result from the pleiotropic effects of other gene(s) at the *S*-locus (Dulberger 1992; Al Wadi and Richards 1993).

More recently, Kurian and Richards (1997) proposed that there are at least seven loci at the distyly supergene in *Primula*. This suggestion was, in part, based on their studies of a curious novel phenotype (P^*) discovered in a hybrid cultivar (*P.* × *tommasinii*). This plant resembles a self-compatible L-morph with a shortened style that produces dimorphic pollen, both sizes of which exhibit the incompatibility phenotype of large-sized pollen of the S-morph. Following a study of the inheritance of the P^* variant, Kurian and Richards (1997) proposed a gene for pollen incompatibility separate from the gene determining pollen size. Richards (2003) subsequently proposed as many as nine linked genes comprising the *Primula* supergene. However, there are no convincing genetic data to support this claim.

Evidence that distyly is determined by a supergene in other heterostylous families is limited and no study has unambiguously documented recombinants arising from controlled crosses. The inheritance and compatibility behaviour of homostyles have been investigated in *Fagopyrum* (Woo et al. 1999; Wang et al. 2005b; Fesenko et al. 2006) and *Turnera* (Shore and Barrett 1985; Barrett and Shore 1987; Tamari et al. 2001) using inter-specific crosses between distylous and self-compatible long homostylous species. In both taxa, the dominance hierarchy among alleles at the *S*-locus is consistent with the *Primula* inheritance pattern (i.e. $S > S^h > s$), where the S^h-allele confers the long homostyle phenotype in S^hS^h and S^hs genotypes, although a second unlinked locus may be involved in *Fagopyrum* (Wang et al. 2005b; Fesenko et al. 2006). The compatibility relations between distylous and homostylous species exhibit a pattern comparable to that observed for *Primula*, with the style of the homostyle retaining the incompatibility phenotype of long styles, and pollen of the homostyle exhibiting the large size and incompatibility phenotype of pollen of the S-morph (Fig. 1.4). The inheritance and compatibility data have been interpreted as evidence that distyly is controlled by a *Primula* type supergene. The apparent S^h allele is inferred to result from recombination within the supergene, giving rise to long homostyles (*gPA/gpa* or *gPA/gPA*) as observed in *Primula* spp. (Figs. 1.4 and 1.5; see also Chap. 14, this volume). However, in *Fagopyrum* and *Turnera*, evidence supports the occurrence of only two loci at the supergene because plants have not been recovered in which pollen size and stamen height are dissociated (Woo et al. 1999; Matsui et al. 2003; Tamari et al. 2005).

There are few cases outside of *Primula* where the inheritance of mutants or putative recombinants has been studied to explore the genetic architecture of distyly. A homostylous somatic mutant arose as a shoot on an otherwise S-morph F_1 plant from the cross between distylous *Turnera subulata* and distylous *T. krapovickasii*. The mutant behaved as if determined by an allele, S^h, at the distyly locus, with the dominance and compatibility relations as observed for long homostyles of *Primula* (Tamari et al. 2005; Fig. 1.4). While clearly not the result of meiotic recombination, its mutational origin and inheritance are consistent with the possibility that a separate mutable gene for style length and associated style incompatibility might

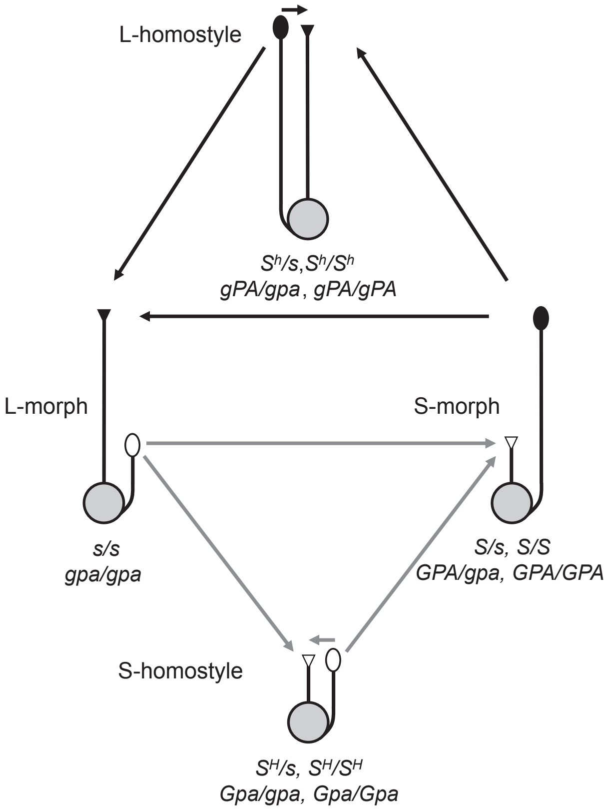

Fig. 1.4 Compatible pollinations between distylous and homostylous morphs. The L- and S-morphs are self-incompatible, whereas the long and short homostyle morphs (L- and S-homostyle, respectively) are self-compatible. Arrows indicate compatible pollinations. Genotypes of the morphs are indicated as alleles at the *S*-locus with the dominance hierarchy $S > S^h, S^H > s$, and as a series of three linked alleles comprising a supergene. For the genotypes illustrated, the S^h allele determines the L-homostyle, while S^H determines the S-homostyle (the superscripts for the alleles conferring homostyly do not imply that one allele is dominant to the other). Both homostyles are incompatible with one another; however, if the homozygous genotypes were able to be crossed, and assuming the supergene model, only S-morph progeny would be produced because of complementation

reside adjacent to a gene(s) determining stamen characteristics. A long homostylous 'mutant' of distylous autotetraploid *T. scabra* exhibited comparable behaviour coupled with tetrasomic inheritance (Tamari et al. 2005). Therefore, outside of *Primula* conclusive evidence of a supergene based on clear documentation of recombination yielding novel floral morphs is lacking. The available genetic data are consistent with the occurrence of two linked loci in *Turnera* and *Fagopyrum*, but are not unequivocal demonstrations of the existence of supergenes.

Supergene control of tristyly is often implied in the literature, although there is actually no direct genetic evidence (Charlesworth 1979; Lewis and Jones 1992;

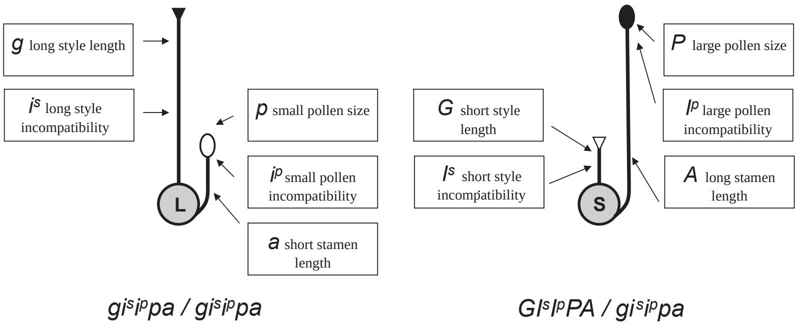

Fig. 1.5 A supergene model for the inheritance of distyly involving five tightly linked genes. In addition to the G/g, P/p and A/a genes, two further genes determining pollen, I^p/i^p, and style, I^s/i^s, incompatibility are included, as often inferred in the literature (see Sect. 1.4). Each gene acts independently to determine the morphological and incompatibility phenotypes of morphs, as indicated by the arrows

Barrett 1993). Because of the occurrence of two stamen levels within tristylous flowers, homostylous variants (referred to as semi-homostyles) are usually characterized by a change in the position of a single stamen level only. However, subsequent selection associated with the evolution of small flowers and self-fertilization can result in both stamen levels in close proximity to stigmas (Ornduff 1972; Barrett 1988). For reasons that still need to be determined, semi-homostylous variants most often involve the M-morph. Under the supergene model, semi-homostyles should be inherited as if determined by a new allele at the *S* or *M* locus (Charlesworth 1979). Genetic analysis of semi-homostyly is limited; however, what data are available [e.g. *Lythrum salicaria* Stout (1925); *Eichhornia paniculata* Fenster and Barrett (1994)] do not indicate recombinational origins, but rather mutations non-allelic to the *S* and *M* loci.

A supergene model for tristyly requires the occurrence of two supergenes, one residing at each of the *S* and *M* loci. Genes within each supergene complex would each exhibit the usual pattern of dominance and epistatic interactions. The difficulty in imagining how supergenes may have evolved in tristylous species led Charlesworth (1979) to invoke pleiotropy and ‘incompatibility gradients’ in her model of the evolution of tristyly. Several lines of evidence support elements of her model. For example, changes in incompatibility response and pollen size are associated with alterations in the position of stamens in tristylous species (Barlow 1913; Weller 1976; Manicacci and Barrett 1995). Regulatory genes may play a role in altering the incompatibility phenotype of pollen within individual flowers and perhaps also in controlling other features of tristyly. If this is true, we see no reason why comparable regulatory genes may not also be involved in controlling elements of the distylous syndrome, especially in cases where distyly has evolved from tristyly, as in *Lythrum* and *Oxalis* (reviewed in Weller 1992).

1.4.3 Mutational Analyses and the Study of Genetic Variants

A useful approach for probing the genetic architecture of heterostyly is to investigate mutants. Under the supergene model, mutant alleles determining novel floral morphs (e.g. homostyles) should map to the *S*-locus and involve predictable phenotypes (Mather 1950; Matsui et al. 2007). In an ongoing experiment, pollen carrying the dominant *S*-allele from homozygous (*SS*) S-morph plants of *Turnera subulata* was X-ray irradiated and used to pollinate L-morph (*ss*) plants (Shore et al. 2006; Labonne and Shore, unpublished data). The cross produced three L-morph mutants and one long homostyle mutant. Although the inheritance of the mutants has yet to be determined, a molecular marker tightly linked to the *S*-locus was deleted in each of the four mutants, suggesting that the mutations occur at the *S*-locus. The occurrence of a long homostyle mutant supports earlier work on *Turnera*, suggesting that distyly is controlled by at least two linked genes.

It is unlikely that a single gene determines all of the heteromorphic features of distylous plants unless it is a complex regulatory gene that resides at the *S*-locus. Alternatively, it seems unreasonable to us that each character of the suite of dimorphic traits results from a separate linked gene. Elements of one possible supergene model are illustrated in Fig. 1.5 (Dowrick 1956; Ganders 1979, Lewis and Jones 1992; Matsui et al. 2003 and Chap. 14, this volume). Could a single regulatory gene at the *S*-locus be responsible for all of the trait differences among the morphs? In *Primula*, *Turnera* and *Fagopyrum*, inheritance data for homostyles appear to discount this possibility but may indicate that at least two (*Fagopyrum* and *Turnera*) and possibly three (*Primula*) linked regulatory loci occur. One of the regulatory loci could determine gynoecial characteristics (style length and incompatibility) and the other androecial characteristics (pollen size, production and incompatibility). These loci could conceivably regulate genes that might be distributed elsewhere in the genome (Fig. 1.6). The capacity of the dominant *S*-allele to directly or indirectly up-regulate genes located outside the *S*-locus has been demonstrated in *Turnera* (Athanasiou et al. 2003; Khosravi et al. 2004; Tamari and Shore 2006) and *Primula vulgaris* (McCubbin et al. 2006). This raises the possibility that ‘incompatibility genes’ may not reside at the *S*-locus, but instead are turned on by signals generated by the *S*-locus (Fig. 1.6). If correct, then it would be difficult to recover mutant alleles at the *S*-locus in which style length and pollen size are separated from their respective incompatibility responses, although non-allelic mutations could be found, as may be the case for a self-compatible plant of the S-morph of *T. subulata* (Shore and Barrett 1986; Athanasiou and Shore 1997).

1.5 Molecular Genetics

Despite the long interest in heterostyly as a plant model system for linking genetics and adaptation, the molecular basis of the polymorphism has yet to be determined. Attempts to find the genes and proteins responsible for heteromorphic SI

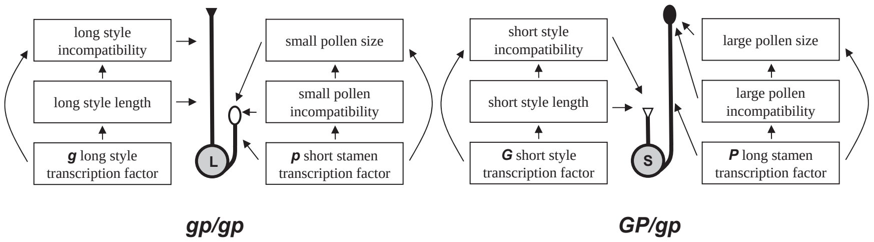

Fig. 1.6 A model of the genetic architecture of distyly that involves morph-limited gene expression. In the model, two closely linked genes (G/g and P/p) occur at the S-locus. G/g encodes a transcription factor responsible for the direct or indirect regulation of genes for gynoecial characters (e.g. style length, style incompatibility), whereas P/p encodes a transcription factor responsible for the direct or indirect regulation of genes for androecial characters (e.g. stamen length, pollen size, pollen incompatibility). The two alleles of each transcription factor may regulate different suites of genes distributed in the genome. The arrows indicate possible patterns of regulation in which the initial transcription factor directly regulates one or more genes, and/or the regulation of one of these genes causes a cascade of regulatory effects on other genes

in distylous species were initiated during the past decade. These included efforts to discover proteins or RNA transcripts unique to pollen and/or styles of the morphs, and genetic mapping and positional cloning of the putative genes determining distyly. Discovery of the genes governing distyly may depend on specific details of how the polymorphism evolved, because the assembly of the polymorphism seems likely to have influenced the underlying molecular mechanism(s). Theoretical models for the evolution of heterostyly may be useful for informing future molecular investigations. As mentioned previously (see Sect. 1.2.1.), there are two widely discussed models for the evolution of distyly (Charlesworth and Charlesworth 1979a; Lloyd and Webb 1992a, b). Although other models have been proposed (Richards 2003; Sakai and Toquenaga 2004), they have attracted little attention, probably because they contain several unlikely assumptions, and we therefore do not consider them further. Below we briefly review relevant features of the Charlesworth and Charlesworth (1979a) and Lloyd and Webb, (1992a, b) models and consider the molecular methods best suited to discover the genes determining distyly, based on predictions of these models.

1.5.1 Theoretical Models and Predictions

Charlesworth and Charlesworth (1979a) used a population-genetic approach to model the evolution of distyly. In their model, the SI system evolves first, followed by reciprocal herkogamy. There is a linkage constraint in which a recessive mutation to a new pollen type arises first, and the population is then invaded by a dominant mutation to a new style type, provided that the mutation occurs at a linked locus. Subsequently, morphological differences evolve enhancing compatible inter-morph pollinations, and there are likely to be linkage constraints involved in their evolution as well. A number of linked genes determine distyly according to this model. Stylar incompatibility is governed by allelic variation at one of the supergene loci, whereas pollen incompatibility results from variation at a linked locus. Likewise, genes for style and stamen length result from allelic variation linked to the incompatibility loci. Fine-scale genetic mapping and positional cloning would appear to be the best approach to test predictions of this model. Under this model all genes that determine the polymorphism reside at the *S*-locus supergene (e.g. Fig. 1.5).

Lloyd and Webb (1992a, b) provide an alternative phenotypic selection model for the evolution of distyly based on the efficacy of cross-pollen transfer. In their model reciprocal herkogamy evolves prior to the evolution of diallelic incompatibility. A variant with reduced style length determined by a dominant mutation invades a population composed of plants exhibiting approach herkogamy, resulting in polymorphism for stigma height. According to the model, higher inter- than intra-morph pollen transfer maintains stylar dimorphism, although the mechanism responsible for promoting disassortative pollination is unclear (Stone and Thomson 1994). There is then selection for anther-height dimorphism and associated ancillary features, which improves the efficacy of inter-morph pollen transfer. Finally, SI (if it develops) evolves gradually as a result of co-adaptation between pollen-tube growth and the styles in which they most commonly grow. Under this scenario, SI could result from genes with morph-limited expression, or the genes involved may be linked to genes for style length and/or anther position. Morph-limited expression of the SI response may also be increased by subsequent selection. That is, mutations arising anywhere in the genome could cause pollen tubes to grow less well in one or the other stylar environment.

In contrast to the Charlesworth and Charlesworth (1979a) model, the genes responsible for distyly in the Lloyd and Webb model could be distributed throughout the genome, although some could also be linked (Lloyd and Webb 1992b, p 198). Lloyd and Webb (1992a, b) predict that SI is unlikely to involve the type of *S*-gene specificities found in homomorphic systems. They also propose that the SI mechanism may be different between the morphs. Genes exhibiting differential expression between the morphs could also be responsible for the regulation of various other features of distyly (Fig. 1.6). Comparing protein or mRNA profiles of the morphs may be the best approach for testing the predictions of the Lloyd and Webb model since this would allow the discovery of expression differences, and possibly allelic variation manifested in proteins or mRNA. Fine-scale genetic mapping and positional cloning of gene(s) at the *S*-locus should lead to the discovery of the gene determining style-length variation, and possibly a regulatory gene(s) responsible for the expression of incompatibility and various ancillary features. Should such a regulatory gene(s) be discovered, it might be possible to find its interacting partners using, for example, chromatin immunoprecipitation assays or in vitro genomic selection (Wang 2005). Below we review progress made in attempts to discover the molecular basis of heterostyly using three main approaches: protein profiles, mRNA expression and genetic localization. See other chapters in this volume (Part B) for accounts of investigations into the molecular basis of homomorphic SI.

1.5.2 Protein Profiles

The first attempts to investigate the molecular basis of heterostyly used methods largely designed to detect protein differences among the morphs. The earliest experiments involved cytochemical staining of tissues (Dulberger 1974), as well as physiological approaches, exploring the effects of style and/or stigma extracts on pollen germination in vitro (reviewed in Gibbs 1986; Dulberger 1992; Chap. 14, this volume). More recent efforts have used protein separation techniques, including isoelectric focussing (IEF), one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and 2D PAGE. Wong et al. (1994) used both SDS-PAGE and 2D PAGE to compare protein profiles of floral organs in distylous *Averrhoa carambola* (Oxalidaceae). They discovered an abundant 72 kDa protein unique to styles of the L-morph but the identity of the protein was not determined. Miljuš-Đukić et al. (2004) compared protein profiles of the L- and S-morphs of *Fagopyrum esculentum* using similar techniques and also found differences between the morphs. Once again the identity or function of proteins was not determined.

Studies of *Turnera subulata* and related species resulted in the discovery of two proteins: putative polygalacturonase and α-dioxygenase, unique to styles of the S-morph (Athanasiou and Shore 1997; Athanasiou et al. 2003; Khosravi et al. 2003, 2004). The proteins were localized to the transmitting tissue of styles and stigmas using immunocytochemistry. The gene encoding the polygalacturonase was linked but distal to the *S*-locus (Athanasiou et al. 2003; Tamari and Shore 2006), while the gene for the α-dioxygenase was unlinked (Khosravi et al. 2004). Using 2D PAGE and mass spectrometry, Khosravi et al. (2006) identified two additional proteins unique to the S-morph of *Turnera*: putative cysteine protease and β-expansin. No confirmation of morph- or style-specificity has yet been undertaken nor are the possible roles of the proteins known.

Recently, protein expression differences have been reported for the first time in pollen from different stamen levels within flowers of a tristylous species (Kalinowski et al. 2007). Using 2D PAGE of proteins extracted from pollen from each of the six style length-stamen level combinations in *Lythrum salicaria*, these workers report surprisingly large differences in the number of proteins present in different morphs. For example, in the most extreme case, they report 177 proteins present in pollen of mid-level anthers of the S-morph that are not represented in pollen of long-level anthers of the S-morph. Further work is needed to corroborate these findings and determine their relevance, if any, to the functioning of tristyly.

1.5.3 mRNA Expression

Two studies have attempted to find transcripts unique to the floral morphs of distylous *Primula vulgaris*. McCubbin et al. (2006) used suppressive subtractive hybridization to discover 11 cDNAs differentially expressed between the morphs. None of these were linked to the *S*-locus and the authors suggest that the genes may be downstream components of developmental pathways, leading to floral dimorphism and/or physiological differences between the morphs. By contrast, Li et al. (2007) used differential display to identify transcripts unique to the floral morphs undertaking an extensive sampling of the transcriptome of a single size class of flower bud. Only one amplification product proved to be differentially expressed and sequencing provided no information on its identity. However, significantly, two of the initial amplification products proved to be linked to the *S*-locus (see Sect. 1.5.4; see also Chap. 14, this volume). These results from *Primula vulgaris* differ with respect to the number of differentially expressed genes found. This may have occurred because McCubbin et al. (2006) used a range of bud sizes and mature tissues in their analysis.

1.5.4 Genetic Localization

Linkage maps were first constructed for distylous species early in the last century (De Winton and Haldane 1935). Today the advent of hypervariable molecular markers allows high-resolution linkage maps to be constructed and, because the markers are typically DNA fragments, they can be used to probe cDNA and/or genomic libraries to discover genes of interest.

Progress towards positional cloning of the *S*-locus has been made in *Fagopyrum*. Aii et al. (1998) constructed a linkage map targeting the *S*-locus. They used 181 F_2 progeny from an inter-specific cross of distylous *Fagopyrum esculentum* with homostylous *F. homotropicum*. Three RAPD markers were linked in coupling with the S^h-allele, the closest at a distance of 0.6 cM. Both Nagano et al. (2001) and Yasui et al. (2004) extended this analysis and discovered AFLP markers closely linked to the *S*-locus. The linked markers should provide useful starting points for high-resolution mapping and positional cloning of *S*-locus genes. A bacterial artificial chromosome (BAC) library has been constructed from *F. homotropicum* that should enable positional cloning (Nagano et al. 2005).

Manfield et al. (2005) discovered a 323 bp RAPD marker tightly linked ($\sim$0.5 cM) in coupling to the *S*-allele in a diploid hybrid horticultural variety of *Primula vulgaris*. They probed a phage λ genomic DNA library and obtained a clone of 8.8 kb, approximately 1 kb of which showed homology to regions of Ty3/gypsy-like retrotransposons. The remainder of the sequence consisted of multiple short repetitive elements. To determine on which side of the *S*-locus the linked marker resides, these authors attempted to amplify the 323 bp fragment from genomic DNA of a long homostyle, with predicted genotype *gPA/gPA*, and a short homostyle, predicted genotype *Gpa/gpa*. The homostyles were developed in a wild *P. vulgaris* genetic background. The 323 bp fragment amplified in the long homostyle but not in the short homostyle. Assuming the homostyles arose by recombination within the supergene, and their genetic backgrounds and the cultivar are similar in the region around the *S*-locus, these results indicate that the 323 bp fragment lies on the *A*-locus side of the supergene, or possibly within the supergene but adjacent to the *P*- or *A*-locus.

Li et al. (2007) used Southern analyses to demonstrate that two differential display products were closely linked to the *S*-locus. One gene, *PvSLL1*, co-segregated with the *S*-locus, while the second, *PvSLL2*, was closely linked to it. *PvSLL1* encodes a putative small transmembrane protein with similarity to an *Arabidopsis* gene of unknown function. These authors used long and short homostyles of *P. vulgaris* to map the location of both genes (as above). The *S*-coupled allele of *PvSLL1* occurred in the homozygous (*gPA/gPA*) long homostyle, while one of the two *s*-coupled alleles was found in the short homostyle. These results suggest that *PvSLL1* lies on the *A*-locus side of the supergene within 0.57 cM of the *S*-locus, or even possibly within the supergene, since no recombinants were found. Further analysis revealed that *PvSLL2* lies on the *G*-locus side of the supergene at a map distance of approximately 1.4 cM. The identification of these closely linked genes and the marker identified by Manfield et al. (2005) will provide valuable probes for screening a BAC library of *Primula* and should enable positional cloning of *S*-locus genes.

In distylous *Turnera subulata*, isozyme loci (*Aco-1* and *Pgd-c*) map 3.3 cM and 1.8 cM, respectively, on either side of the *S*-locus (Athanasiou and Shore 1997; Athanasiou et al. 2003). Labonne et al. (2007) demonstrated significantly greater female meiotic recombination rates in *T. subulata*, expanding the map distance between the two isozyme loci approximately 4–6-fold. Further mapping efforts involved greater precision because of higher recombination rates in female meiosis. Labonne et al. (2008) obtained a fine-scale map of the *S*-locus region based upon 736 backcross progeny. Two closely linked molecular markers on opposite sides of the *S*-locus have been mapped, as well as two co-segregating markers. Chromosome walking has been initiated to identify gene(s) that reside at the *S*-locus in *T. subulata*.

As with the homomorphic SI systems (Charlesworth 2006), balancing selection has likely maintained the distylous polymorphism over long evolutionary time scales. As such, the application of molecular population genetic approaches should aid in identifying the target(s) of balancing selection and testing whether there is recombination suppression in the *S*-locus region. Genes comprising, or tightly linked to, the *S*-locus identified through mapping studies should show elevated nucleotide diversity (i.e. the footprint of balancing selection), as has been demonstrated for homomorphic SI systems (Kamau and Charlesworth 2005; Charlesworth 2006). For genes less tightly linked, infrequent recombination over evolutionary time will erode this diversity (Charlesworth 2006). While conclusive proof of the function of genes at the *S*-locus can best be demonstrated using transformation and knockout strategies, molecular population genetics will be important in identifying putative *S*-locus genes.

1.6 Concluding Remarks

Heterostyly continues to fascinate evolutionary biologists interested in the genetics, evolution and function of adaptations. The polymorphisms illustrate how simply inherited developmental changes in the positions of plant sexual organs have important consequences for the pollination and mating biology of populations. Unlike homomorphic SI, only a small number of mating phenotypes can be maintained in populations because of the constraints in achieving multiply-segregated sites for pollen deposition on animal pollinators. The three floral morphs in tristylous populations probably represent the upper limit for mating groups that can be achieved for sexual polymorphisms that function to promote cross-pollination by the geometry of pollinator contacts.

Our review has highlighted recent work on the comparative biology, ecology and genetics of heterostyly. Over the past few decades, studies on a wider range of heterostylous species have broadened our understanding of the polymorphisms and demonstrated that considerable variation in expression occurs among taxonomic groups. This variation should caution against overgeneralization of the *typical features* of heterostyly, based on studies of models systems such as *Primula*. Comparisons of variation in heterostylous traits, particularly the occurrence of diallelic incompatibility and ancillary pollen and stigma polymorphisms, in lineages that differ in the number of heterostylous species may be instructive. The expression of the heterostylous syndrome in genera with single isolated heterostylous species could provide clues on the early stages in the assembly of the polymorphism and shed light on models of the evolution of heterostyly.

A major theme in the literature concerns the evolutionary pathways responsible for the buildup and breakdown of heterostyly (Ganders 1979; Weller 1992). Evidence for character state transitions has come largely from comparative arguments (Baker 1966; Ornduff 1966) or population-level studies of intra-specific variation (Piper et al., 1986; Barrett et al. 1989; Weller et al. 2007). Future work using phylogenies and character reconstructions are needed to provide convincing evidence linking observed microevolutionary changes to putative macroevolutionary patterns. Boraginaceae, Menyanthaceae, Oxalidaceae, Plumbaginaceae and Rubiaceae are groups that contain particularly high sexual-system diversity and warrant serious attention by comparative biologists interested in the evolution of reproductive systems.

As evident from other chapters in this volume, progress on determining the genes controlling heterostyly has lagged behind research on homomorphic incompatibility. Lewis and Jones (1992) suggested that the incompatibility genes in heterostylous species should be of first priority for molecular dissection, predicting that they would have considerable output of transcribed mRNA and translated protein. However, subsequent work on *Primula* did not find abundant and/or differentially expressed mRNA encoded by genes linked to the *S*-locus (McCubbin et al. 2006 and this volume; Li et al. 2007). Similarly, identifying *S*-locus proteins distinguishing the morphs in distylous *Fagopyrum* (Miljuš-Đukić et al. 2004) and *Turnera subulata* (Athanasiou et al., 2003; Khosravi et al., 2004) has also proven elusive. Hence, collectively there is no compelling evidence for highly abundant transcripts or proteins governing diallelic incompatibility.

Genes regulating floral development may exhibit differential expression, obscuring the signal of incompatibility genes and making their identification more difficult (McCubbin et al. 2006). Alternatively, there may be no abundantly expressed incompatibility genes and/or proteins, because, as Lloyd and Webb (1992a, b) suggested, incompatibility may largely result from genes elsewhere in the genome affecting pollen-tube growth. The up- vs. down-regulation of genes reported by McCubbin et al. (2006) and the observed protein differences between styles of the floral morphs may be key elements in the expression of diallelic incompatibility. Identification of the genes controlling heterostyly will help to illuminate theoretical models for the evolution of the polymorphism. When this is achieved, the long quest for determining the causal relations between genes, form and adaptive function in heterostylous plants will have finally been achieved.

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