Tansley insight

Monkeyflowers (*Mimulus*): new model for plant developmental genetics and evo-devo

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Summary

Monkeyflowers (*Mimulus*) have long been recognized as a classic ecological and evolutionary model system. However, only recently has it been realized that this system also holds great promise for studying the developmental genetics and evo-devo of important plant traits that are not found in well-established model systems such as Arabidopsis. Here, I review recent progress in four different areas of plant research enabled by this new model, including transcriptional regulation of carotenoid biosynthesis, formation of periodic pigmentation patterns, developmental genetics of corolla tube formation and elaboration, and the molecular basis of floral trait divergence underlying pollinator shift. These examples suggest that *Mimulus* offers ample opportunities to make exciting discoveries in plant development and evolution.

I. Introduction

The wildflower genus *Mimulus* (monkeyflowers) has been widely recognized as a classic ecological and evolutionary model system (Hiesey *et al.*, 1971; Wu *et al.*, 2008) in studying local adaptation (Lowry *et al.*, 2009; Kooyers *et al.*, 2015; Hendrick *et al.*, 2016; Selby & Willis, 2018), speciation (Ramsey *et al.*, 2003; Streisfeld *et al.*, 2013; Zuellig & Sweigart, 2018), species range limits (Angert & Schemske, 2005; Sheth & Angert, 2018) and plant–pollinator interactions (Schemske & Bradshaw, 1999; Holmquist *et al.*, 2012). What is less well-known, however, is that this system also holds great promise for studying the developmental genetics of important plant traits that are not found in well-established model systems such as Arabidopsis (e.g. flower pigmentation patterns, corolla tubes, underground rhizomes, tolerance to salt, heavy-metal or serpentine soils, and geothermal environments). Additionally, the *c.* 170 species in the genus exhibit tremendous phenotypic diversity (Fig. 1; Box 1), providing an excellent platform for detailed molecular dissection of the genetic bases and developmental mechanisms of phenotypic diversification – a central goal of evo-devo. It is my hope that this short review will introduce the *Mimulus* system to researchers beyond the ecology and evolutionary biology communities (e.g. plant molecular biologists, physiologists, biochemists, developmental biologists), who may be

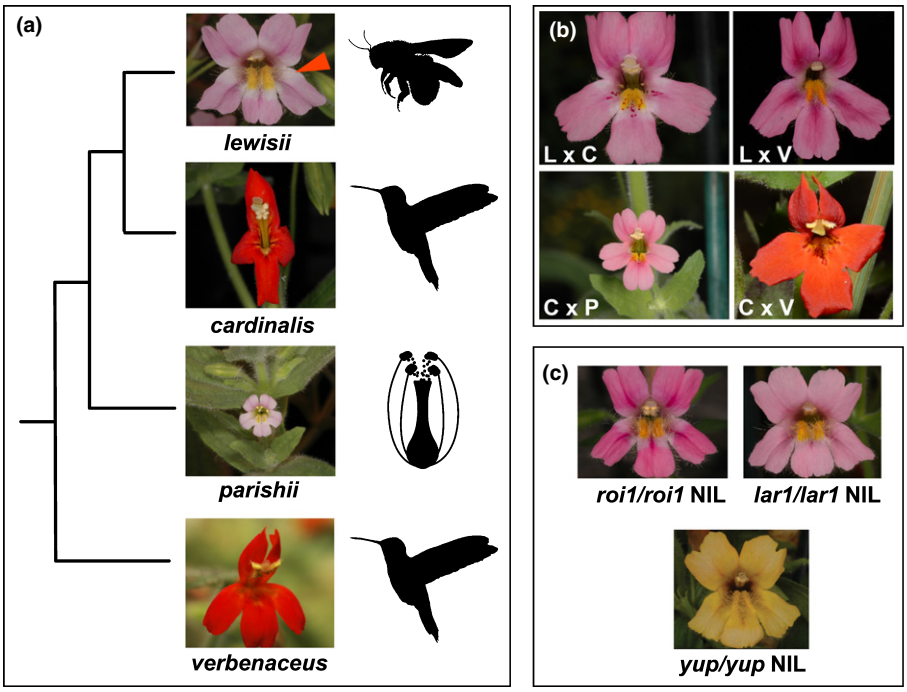


Fig. 1 The *Mimulus lewisii* species complex. (a) Flower phenotypes and pollination syndromes of the four focal species (pollination syndromes illustrated by Qiaoshan Lin). Phylogenetic relationships are based on Beardsley *et al*. (2003). The red arrow indicates the light areas around the corolla throat. (b) Flower phenotypes of F_1 hybrids (L, *M. lewisii*; C, *M. cardinalis*; V, *M. verbenaceus*; P, *M. parishii*). (c) Near-isogenic lines (NILs) of the three flower color loci in the *M. lewisii* background. All flower images are scaled in proportion to the actual flower sizes.

interested in using this wonderful and versatile model to address various long-standing questions in plant biology.

II. The system

Mimulus (family Phrymaceae) is a typical member of Lamiales, a large order containing > 20 000 species (Refulio-Rodriguez & Olmstead, 2014), including the classic genetic model system *Antirrhinum* (family Plantaginaceae). Closely related to Lamiales is the order Solanales, which contains another genetic model, *Petunia* (family Solanaceae). All three genera produce flowers with petals fused into a corolla tube, a defining character of asterids, one of the two major clades of eudicots. By contrast, genera in the other eudicot clade, rosids (e.g. *Arabidopsis*), usually bear flowers with completely separate petals. Although *Antirrhinum* and *Petunia* have a long history in developmental genetics studies, largely due to their endogenous, active transposons that are convenient agents for mutagenesis and subsequent gene isolation (Schwarz-Sommer *et al*., 2003; Vandenbussche *et al*., 2016), *Mimulus* complements these previously established asterid systems for its relative ease in chemical mutagenesis and *in planta* stable transformation.

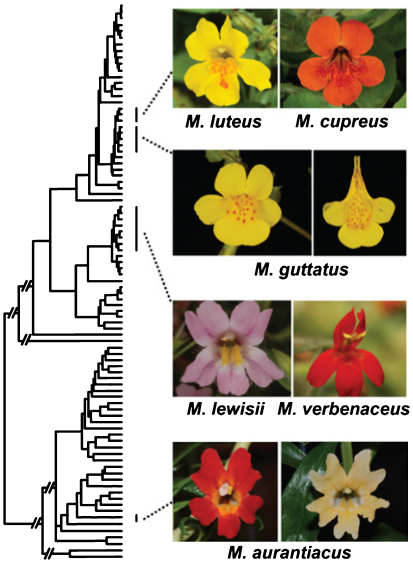
Among the several *Mimulus* species that are potentially suitable models for plant developmental genetics and evo-devo studies (Box 1), the best developed to date is the *M. lewisii* complex, including the bumblebee-pollinated *M. lewisii*, hummingbird-pollinated *M. cardinalis* and *M. verbenaceus*, and self-pollinated *M. parishii* (Fig. 1a). Despite being dramatically different in flower and leaf phenotypes, as well as eco-physiological adaptations, these species are genetically so similar (> 97% identical in coding regions) that they can be readily crossed with hand-pollination in the glasshouse to produce fertile offspring (Fig. 1b). These species have several features that greatly facilitate genetic analysis, including high fecundity (up to 1000 seeds per flower), short generation time (2.5–3 months), and small genome size (*c.* 500 Mb). In the past several years, a number of sophisticated genetic resources and functional tools have been developed for these species, including: (1) an efficient *Agrobacterium*-mediated, *in planta* stable transformation protocol that allows for transgenic experiments to rigorously characterize gene function (Yuan *et al*., 2013a); (2) a transient gene expression assay by leaf agroinfiltration to rapidly determine subcellular protein localization and to test protein–DNA and protein–protein interactions (Ding & Yuan, 2016); and (3) large-scale ethyl methanesulfonate (EMS) mutant libraries that facilitate genetic dissection of developmental programs and regulatory networks (Yuan *et al*., 2013b, 2014; Sagawa *et al*., 2016; Ding *et al*., 2018a,b). In the rest of this paper I will briefly describe a few exemplar research areas where these resources and tools have enabled fruitful investigations.

III. Regulation of carotenoid pigmentation

Carotenoids are yellow, orange and red pigments that contribute to the beautiful colors and nutritive value of many flowers (e.g. daffodils, daylilies, sunflowers) and fruits (e.g. oranges, tomatoes, mangos). They also serve an important function in the ecology and evolution of plants by attracting pollinators and seed dispersers. The incredible diversity of carotenoid pigmentation patterns in angiosperm flowers and fruits is largely determined by differential

Box 1 Other *Mimulus* species that are potential models for plant developmental genetics or/and evo-devo studies

Shown on the left are the *M. guttatus*, *M. luteus* and *M. aurantiacus* species complexes as well as their phylogenetic positions relative to the *M. lewisii* complex. The phylogenetic tree is adapted from Grossenbacher & Whittall (2011). All species shown in the figure are amenable to stable transformation. Shown on the right are selected traits that are particularly suitable to study using the corresponding species complex. Note that a recent taxonomic treatment has split the genus *Mimulus* into at least three genera, and has placed the *M. guttatus*, *M. luteus* and *M. lewisii* complexes in the genus *Erythranthe*, and the *M. aurantiacus* complex in the genus *Diplacus* (Barker *et al.*, 2012). However, the conventional nomenclature scheme is followed in this review for continuity with the large body of previous work on the ecology and evolution of this group.



***M. luteus* complex:**
Parallel gains of petal pigmentation (Cooley *et al.*, 2011);
Phenotypic novelty of experimental hybrids (Cooley *et al.*, 2009)

***M. guttatus* complex:**
Salt tolerance (Lowry *et al.*, 2009);
Copper tolerance (Wright *et al.*, 2013);
Serpentine soil adaptation (Selby & Willis, 2018);
Geothermal adaptation (Hendrick *et al.*, 2016);
Leaf shape (Ferris *et al.*, 2015)

***M. aurantiacus* complex:**
Gains of petal pigmentation (Stankowski & Streisfeld, 2015);
Nectar–microbe interactions (Vannette & Fukami, 2018)

expression of the carotenoid biosynthetic genes (Moehs *et al.*, 2001; Ha *et al.*, 2007; Yamamizo *et al.*, 2010), yet no transcription factors regulating carotenoid pigmentation during flower development had been reported before the analyses of *M. lewisii* mutants (Sagawa *et al.*, 2016; Stanley *et al.*, 2017).

The ventral (lower) petal of *M. lewisii* flowers has two yellow ridges that are pigmented by carotenoids (Fig. 2a), acting as nectar guides for bumblebee pollinators (Owen & Bradshaw, 2011). Loss-of-function mutations in the *REDUCED CAROTENOID PIGMENTATION 1* (*RCP1*) and *RCP2* genes cause decreased carotenoid concentration (Fig. 2b,c) and coordinate transcriptional downregulation of the entire carotenoid biosynthetic pathway (Sagawa *et al.*, 2016; Stanley *et al.*, 2017). Independent *rcp2* alleles also have been isolated by EMS mutagenesis of the closely related *M. verbenaceus* (Fig. 2e,f). *RCP1* and *RCP2* encode an R2R3-MYB and a tetratricopeptide repeat (TPR) protein, respectively. The *rcp2* mutant also shows abnormal chromoplast development, suggesting an indirect role in the transcriptional regulation of carotenoid biosynthetic genes, likely through chromoplast-to-nucleus retrograde signaling (Stanley *et al.*, 2017). Another mutant, *yellow expanded* (*yex*), shows enhanced carotenoid pigmentation and expanded yellow areas (Fig. 1d), indicating that a repression mechanism must be operating in and near the nectar guides. Identification of the causal genes of *yex* and additional EMS mutants (not shown here) and sorting out their genetic relationships with *RCP1/2* will help to elucidate the regulatory network of floral carotenoid pigmentation.

IV. Formation of periodic pigmentation patterns

Many organisms exhibit interesting pigmentation patterns (e.g. zebra stripes, leopard spots). Formation of such periodic patterns in biological objects is often explained by Turing's reaction-diffusion (RD) model (Turing, 1952). The essence of RD-based models is an interacting network that contains a local autocatalytic feedback loop and a long-range inhibitory feedback loop involving activators and repressors (Meinhardt & Gierer, 2000; Kondo & Miura, 2010; Davies *et al.*, 2012; Green & Sharpe, 2015). Computer simulations using RD models with different parameter values can generate a wide variety of periodic pigmentation patterns that are remarkably similar to those found in real organisms (Kondo & Miura, 2010). However, the molecular identities of hypothetical activators and repressors that fulfill the RD model requirements have remained elusive, although putative activator–repressor pairs have been proposed for several periodic patterns other than pigmentation (reviewed in Marcon & Sharpe, 2012).

Recently, an activator–repressor pair has been identified in *M. lewisii* (Ding *et al.*, 2018a), responsible for the formation of the

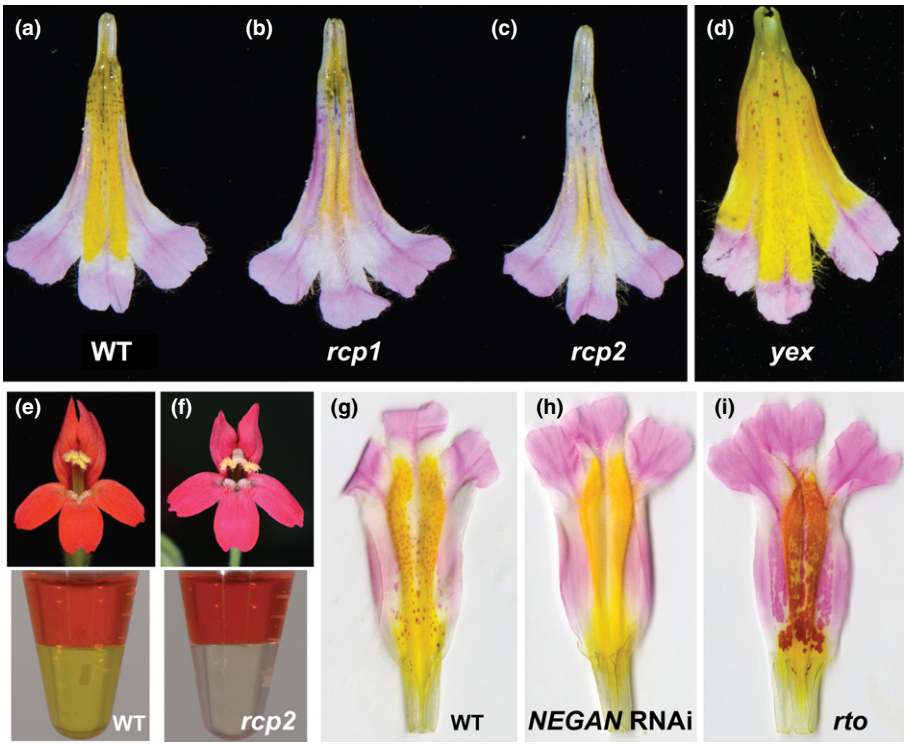


Fig. 2 Floral pigmentation mutants. (a–d) Dissected corolla of the *Mimulus lewisii* wild-type (WT) and three carotenoid pigmentation mutants. The ventral petal has two yellow ridges as nectar guides. (e, f) Wild-type (WT) *M. verbenaceus* (e) and an *rcp2* mutant (f). Upper row: face view of the flower. Lower row: separation of anthocyanins (upper layer) and carotenoids (lower layer). The red color of WT *M. verbenaceus* petals is due to a combination of anthocyanins and carotenoids, as in *M. cardinalis*. (g–i) The anthocyanin spots on the yellow background (g) are abolished in the *NEGAN* RNAi lines (h) and are expanded into large patches in the *rto* mutant (i).

fine anthocyanin spots on the yellow background of the nectar guides (Fig. 2g). The activator, NECTAR GUIDE ANTHOCYANIN (NEGAN), is a typical anthocyanin-activating R2R3-MYB that interacts with a bHLH and a WD40 protein, forming a regulatory protein complex (Davies *et al.*, 2012; Yuan *et al.*, 2014). The repressor, RED TONGUE (RTO), is closely related to a group of R3-MYBs that are known to repress anthocyanin biosynthesis (e.g. *Petunia* MYBx and *Arabidopsis* CAPRICE) by competing with the anthocyanin-activating R2R3-MYB for the limited supply of the bHLH co-activators (Zhu *et al.*, 2009; Albert *et al.*, 2014; Ding *et al.*, 2018a). Downregulation of *NEGAN* expression *via* RNA interference abolishes anthocyanin production in the nectar guides (Fig. 2h), whereas the loss-of-function *rto* mutant causes massive expansion of anthocyanin pigmentation from fine spots to large patches (Fig. 2i). Further transgenic experiments and gene expression analyses demonstrated that this two-component system seems to fit the RD model precisely: the activator, NEGAN, is self-activating and also activates the expression of the repressor, RTO; RTO competes with NEGAN, thereby inhibiting its activity, and can move from the source cell to neighboring cells (Yuan *et al.*, 2014; Ding *et al.*, 2018a). The same NEGAN–RTO network also operates in *M. guttatus*, explaining the formation of the red nectar guide spots (Box 1) (Ding *et al.*, 2018a). Given the ease of experimental manipulations of these species, future studies can be focused on developing live imaging techniques to track pigment production and protein movement in real time, determining whether other mechanisms (e.g. positional information) act upstream of or in parallel with the RD model (Green & Sharpe, 2015), building quantitative models based on the kinetics of NEGAN and RTO in *M. lewisii* and *M. guttatus*, and then using these models to explain natural variation of periodic pigmentation patterns in other species.

V. Developmental genetics of corolla tube formation and elaboration

The corolla tube is interesting from both a developmental and an evolutionary perspective. As a compound organ resulting from union of individual petal primordia, it may represent a developmental path distinct from that of typical vegetative morphogenesis (Verbeke, 1992). As an important component of the enormous diversity of flower morphology in > 80 000 sympetalous species, the corolla tube facilitates many specialized plant–pollinator interactions (e.g. hummingbirds, hawkmoths, nectar bats), which in turn drives rapid diversification of floral forms and plant speciation (Paudel *et al.*, 2015; Lagomarsino *et al.*, 2016). Yet very little is known about the genetic control of the formation of the corolla tube or its subsequent elaboration (e.g. in length, width, curvature).

Analysis of two *M. lewisii* mutants with unfused petals, *flayed1* and *flayed2* (Fig. 3), has provided new insights into the developmental mechanism of corolla tube formation. *flayed1* and *flayed2* are loss-of-function alleles of *ARGONAUTE7*

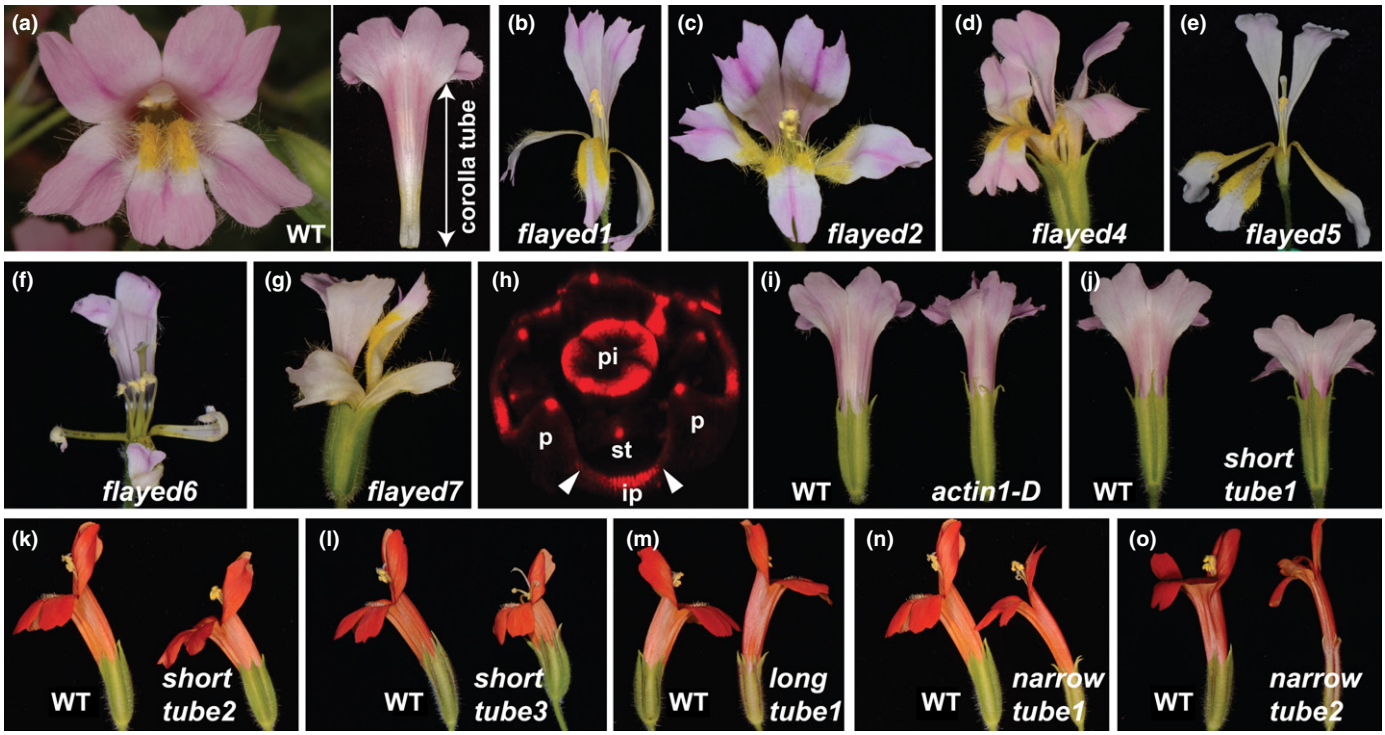


Fig. 3 Corolla tube mutants. (a) Wild-type (WT) *Mimulus lewisii*. Left panel, face view of the corolla; right panel, back view. (b–g) *Mimulus lewisii* mutants with unfused corollas. (h) Patterns of auxin distribution in a developing *M. lewisii* corolla bud (0.5 mm in diameter), as reflected by the *DR5rev:mRFPer* reporter signal. The white arrow heads demarcate the synchronized growth zone encompassing the marginal meristematic cells at the base of the petal primordia and the inter-primordial cells. p, petal; ip, inter-primordial region; st, stamen; pi, pistil. (i, j) *Mimulus lewisii* mutants with altered corolla tube width or length. (k–o) *Mimulus verbenaceus* mutants with altered corolla tube length or width.

(*AGO7*) and *SUPPRESSOR OF GENE SILENCING 3* (*SGS3*), respectively (Ding *et al.*, 2018b). As critical components of the tasi-RNA biogenesis pathway, both AGO7 and SGS3 are necessary to produce *TAS3*-derived small RNAs that repress *AUXIN RESPONSE FACTOR 3* (*ARF3*) and *ARF4* (Peragine *et al.*, 2004; Yoshikawa *et al.*, 2005). As a result, the *flayed1/2* mutants have greatly reduced auxin distribution in developing corolla buds, which prevents the bases of petal primordia from expanding laterally and the inter-primordial regions from growing upward, leading to separated petals instead of a corolla tube (Ding *et al.*, 2018b). In conjunction with the patterns of auxin localization in the wild-type (Fig. 3h), these results suggest that the auxin-directed synchronized growth between the bases of the petal primordia and the inter-primordial regions plays a central role in corolla tube formation (Ding *et al.*, 2018b).

Study of another *M. lewisii* mutant, *act1-D* (Fig. 3i), led to the finding that a dominant negative mutation in the 'housekeeping' actin gene causes substantial decrease in corolla tube width but no change in tube length. This morphological change is mediated by a combination of decreased epidermal cell width and a reduced number of lateral cell divisions (Ding *et al.*, 2017). An important implication of these results is that cytoskeleton dynamics are probably key to understanding corolla tube elaboration. Given the availability of many additional corolla tube mutants in both *M. lewisii* and *M. verbenaceus* (Fig. 3d–g,j–o) and the ease of bulk segregant analysis to identify mutant genes in this system (Yuan *et al.*, 2013b), it is not difficult to envision that *Mimulus* will likely play a major role in elucidating the genetic network(s) controlling corolla tube formation and elaboration, a pre-requisite for understanding the origin of corolla tube in the common ancestor of asterids and the developmental mechanisms for its subsequent diversification. Perhaps one day this information will even enable us to engineer a sympetalous *Arabidopsis* plant.

VI. Molecular basis of floral trait variation underlying pollinator shift

The pollinator-mediated reproductive isolation between *M. lewisii* (bumblebee-pollinated) and *M. cardinalis* (hummingbird-pollinated) represents a classic example in speciation studies (Bradshaw & Schemske, 2003; Ramsey *et al.*, 2003). Flower color was shown to play a major role in pollinator discrimination between the two species (Schemske & Bradshaw, 1999). The pale pink color of *M. lewisii* results from a low concentration of pink anthocyanins and absence of yellow carotenoids (except in the nectar guides; Fig. 1a). The red color of *M. cardinalis* is produced by a combination of high anthocyanin and carotenoid content. The combination of three loci explains much of the flower color difference between the two species (Hiesey *et al.*, 1971): *ROSE INTENSITY1* (*ROI1*) accounts for the anthocyanin content difference in the petal lobe; *Light Areas1* (*LAR1*) is responsible for the presence vs absence of the white region around the corolla throat (red arrowhead in Fig. 1a); and *YELLOW UPPER* (*YUP*) is

responsible for the presence vs absence of yellow carotenoids in the petal lobe. At all three loci, the *M. lewisii* allele is dominant over the *M. cardinalis* allele, and near-isogenic lines have been bred by introgressing the *M. cardinalis* allele into the *M. lewisii* background to isolate the phenotypic effect of each locus (Fig. 1c).

The causal genes underlying *ROI1* and *LAR1* have been identified by fine-scale, recombination-based genetic mapping (Yuan *et al.*, 2013a, 2016). *ROI1* encodes an anthocyanin-repressing R3-MYB, similar to RTO, and is specifically expressed in the petal lobe. *LAR1* encodes a subgroup-7 R2R3-MYB that activates flavonol biosynthesis preferentially around the corolla throat. Flavonol biosynthesis competes with the anthocyanin biosynthetic pathway for the same substrates, which leads to the acyanic ring in *M. lewisii* flowers. For both *ROI1* and *LAR1*, it is the lack of gene expression due to *cis*-regulatory changes in *M. cardinalis* that explains the recessive alleles. However, the causal mutations at these loci have yet to be pinpointed. Genetic mapping of *YUP* and other pollinator-associated floral traits (petal reflexing, stamen and pistil length, nectar volume) is still in progress. The *M. lewisii* species complex not only offers a rare opportunity to dissect the genetic bases and developmental mechanisms of floral trait divergence underlying pollinator shift between two sister species, gene by gene, mutation by mutation, but also allows us to study the switch from outcrossing to self-pollination (Fig. 1a; Fishman *et al.*, 2015), one of the most common evolutionary transitions in angiosperms (Barrett, 2002).

VII. Outlook

In addition to the *M. lewisii* complex, at least three other species complexes in the genus (Box 1) share a suite of advantageous features as found in the *M. lewisii* complex: small genome size, short generation time, high fecundity and, most importantly, amenability to stable transformation (Susič *et al.*, 2014; Ding *et al.*, 2018a; A. Cooley, pers. comm.). These species complexes are favorable systems to study a variety of interesting traits, ranging from salt and copper tolerance to serpentine soil and geothermal adaptations, from phenotypic novelty in experimental hybrids to nectar–microbe interactions (Box 1). Taken together, I hope these examples have made clear that *Mimulus* offers ample opportunities to make exciting discoveries in plant development, physiology and evolution, and that there is a wide-open niche for researchers with various expertise to take advantage of this versatile model system.

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