Evolution of flower color pattern through selection on regulatory small RNAs

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Small RNAs (sRNAs) regulate genes in plants and animals. Here, we show that population-wide differences in color patterns in snapdragon flowers are caused by an inverted duplication that generates sRNAs. The complexity and size of the transcripts indicate that the duplication represents an intermediate on the pathway to microRNA evolution. The sRNAs repress a pigment biosynthesis gene, creating a yellow highlight at the site of pollinator entry. The inverted duplication exhibits steep clines in allele frequency in a natural hybrid zone, showing that the allele is under selection. Thus, regulatory interactions of evolutionarily recent sRNAs can be acted upon by selection and contribute to the evolution of phenotypic diversity.

A convenient system for studying selection in natural populations is afforded by hybrid zones, where closely related species or populations come into contact (1). Such a hybrid zone has been described for two subspecies of *Antirrhinum majus* (snapdragon) that differ in flower color (2), a trait involved in pollinator attraction (3-7). Both subspecies are pollinated by bees but have alternate patterns for guiding flower entry: *A. m. pseudomajus* flowers are magenta, with a patch of yellow highlighting the bee entry point (Fig. 1A), whereas *A. m. striatum* flowers are yellow and magenta veins at the entry point (Fig. 1B). The magenta and yellow flower color intensities show sharp clines at a hybrid zone (2) where the subspecies come into contact. Production of magenta is regulated by *ROSEA* (*ROS*) and *ELUTA* (*EL*) (8-10). *ROS* encodes a MYB-like transcription factor that promotes anthocyanin biosynthetic gene expression in *A. m. pseudomajus* and exhibits a steep cline in allele frequencies at the hybrid zone (2, 9). Distribution of yellow pigment is regulated by *SULF* (Fig. 1, B and C), which represses production of the yellow flavonoid aurone in *A. m. pseudomajus* (Fig. 1D) (2, 9, 10). Here, we study the molecular nature of *SULF*.

To isolate *SULF*, we first mapped it to an interval of ~3 Mb on chromosome 4 by sequencing pools of *sulf* and *SULF* phenotypes from a segregating population (fig. S1). In parallel, we carried out a transposon mutagenesis experiment in *A. majus* (*SULF*) and isolated a mutant, *sulf-660*, that was both somatically and genetically unstable (fig. S2 and supplementary materials). Comparing the genome sequence of *sulf-660* and its revertants revealed a single insertion site, within the mapped region of *SULF*, specific to *sulf-660*. Three independent revertants had different excision footprints at this site, confirming that the transposon was responsible for the *sulf* phenotype (fig. S2B).

Basic Local Alignment Search Tool searches of the sequence flanking the transposon insertion site revealed regions of 74 to 88% nucleotide sequence identity to *A. majus* chalcone 4′-O-glucosyltransferase (*Am4CGT*), which encodes an enzyme involved in synthesis of the yellow pigment aurone (fig. 2A and table S1) (11). The regions of *Am4CGT* homology were organized as an inverted duplication in the *A. majus* *SULF* genome. Both the left and right arms of the duplication carried deletions relative to intact *Am4CGT*, suggesting they had independently degenerated from a more complete precursor. A contiguous region of inverted homology between the left and right arms spanned a ~500-bp region (red arrows, Fig. 2A), separated by a ~600-bp spacer region, which contained the transposon insertion site of *sulf-660*. Phylogenetic analysis indicated that the *SULF* inverted repeats were likely generated from *Am4CGT* recently in the evolution of the *Antirrhinum* lineage (fig. 2B and fig. S3).

To determine whether the inverted duplication at *SULF* might be under selection, we compared *A. m. pseudomajus* and *A. m. striatum* populations sampled from either side of a hybrid zone. Polymerase chain reaction (PCR) using oligos flanking the inverted repeats gave bands in the range 1.5 to 2.5 kb for all individuals from the *A. m. pseudomajus* (*n = 96*) but not the *A. m. striatum* populations (*n = 95*), suggesting that the inverted duplication was present at higher frequency in *A. m. pseudomajus* (fig. S4). Sequencing pools of ~50 individuals from each population revealed reduced depth of sequence for *A. m. striatum* compared with *A. m. pseudomajus* over a ~145-kb region around *sulf*, suggesting that *A. m. striatum* carried deletions relative to *A. m. pseudomajus* in this chromosome region (Fig. 2C).

This conclusion was supported by PCR amplification assays using a range of oligos. Deletion alleles were also observed in resequenced individuals, including a 1.3-kb deletion that removed the left arm of the inverted repeat and part of the spacer sequence in *A. m. striatum*. Thus, the inverted duplication present in *SULF* of *A. m. pseudomajus* is absent or at low frequency in *A. m. striatum* populations, further demonstrating the requirement for the inverted duplication for *SULF* function.

Single-nucleotide polymorphisms (SNPs) in a ~300-kb interval containing *SULF* showed steep clines in allele frequency (Fig. 2D and fig. S5) centered at the same geographic location as clines for *ROS* and flower color (2). SNPs sampled from other positions along chromosome 4 either showed no clines or showed clines centered at different geographic locations (Fig. 2D and fig. S5). The significance of the clines at *SULF* was confirmed by comparing DNA sequences from pools of individuals sampled from a transect covering ~20 km on either side of the hybrid zone. Of the ~7 × 10⁵ polymorphic SNPs on the *SULF* chromosome, 99% showed no allele frequency differences across the transect, and of those that did, more than 99% did not yield steep clines aligned with *ROS*. Thus, there is likely to be strong selection acting on *SULF*.

The coincidence of the *SULF* and *ROS* clines suggests that these loci interact. In *A. m. pseudomajus*, where *ROS* confers magenta color, *SULF* could be favored because it restricts yellow to create a contrasting highlight at the bee entry point (fig. 1A). In *A. m. striatum*, where *ROS* confers reduced magenta intensity for much of the flower, *SULF* could be favored because it confers both a striking yellow color and a contrasting background to the magenta veins (Fig. 1B). Thus, selection acting on different allele combinations at *SULF* and *ROS* allows alternate floral guides to be maintained on either side of a hybrid zone. The situation is comparable to selection acting on loci controlling yellow and red coloration of mimetic patterns in *Heliconius* butterflies (12, 13).

Given the structure of the inverted duplication at *SULF* and its homology to *Am4CGT*, we hypothesized that *SULF* represses *Am4CGT* and thus restricts yellow flower color via regulatory
**Fig. 1. Flower color pattern phenotypes.** Flower face (left) and side (right) views of *A. majus* (*A. m.*) species, showing lower ventral (V), lateral (L), and upper dorsal (D) lobes. Bee vision is sensitive to both yellow and the blue components of magenta reflectance. (A) *A. m. pseudomajus*. Magenta with yellow highlight at the bee entry point. (B) *A. m. striatum*. Yellow with magenta highlights. (C) Flowers from plants with *ros EL* from *A. m. striatum* (*ros EL* and *SULF* from *A. m. pseudomajus* (*SULF P*). (D) Schematic showing the pathways to anthocyanin and aurone pigments. Chalcone synthase, CHS; chalcone isomerase, CHI; *A. m. chalcone 4′-O-glucosyltransferase, Am4′CGT; A. m. aureusidin synthase, AmAS1.

**Fig. 2. SULF locus shows homology to Am4′CGT and signatures of selection.** (A) SULF inverted duplication. Organization of Am4′CGT is shown twice (gray arrows) to indicate regions of homology with SULF (CDS, coding sequence). The left and right inverted repeats at SULF (red arrows) flank the transposon insertion site of sulf-660 (black triangle). (B) Maximum likelihood phylogeny of CGT-related DNA sequences from *Antirrhinum majus* (red), *Mimulus guttatus* (black), and *Linaria vulgaris* (blue). Bootstrap support for nodes with >85% support (red circles, scaled by strength). For extended clade, see fig. S2. (C) Plot of *A. m. striatum* sequence coverage normalized against *A. m. pseudomajus* for pools located at either end of the hybrid zone. Bars indicate genes, with SULF locus in red. Double-headed arrow shows region underrepresented in *A. m. striatum*. Positions of KASP SNPs used forcline analysis (blue dots). (D) Clines for KASP (Kompetitive Allele Specific PCR) markers across the hybrid zone transect. SNP index and chromosome position is indicated above each plot. Markers from SULF show steep clines at the hybrid zone, aligned with clines for ROS1 (right). Markers further away from SULF either show no clines (two examples shown) or clines centered at other geographic locations (fig. S4).
small RNAs (sRNAs). To determine whether SULF generated sRNAs, sRNA libraries were prepared from petals of A. majus SULF and sulf-660. The biggest differences in sRNA abundance mapped to the SULF inverted repeats and corresponded to predominantly 21-nucleotide oligomers (Fig. 3, A and B). RNA blots probed with SULF confirmed that sRNAs from the inverted repeat were present in sulf and absent in sulf genotypes, including A. m. striatum (Fig. 3C and fig. S6). The sRNAs likely derive from processed transcripts predicted to generate long-foldback hairpin RNAs (fig. S7).

If the sRNAs generated by SULF restrict yellow pigmentation by targeting Am4’CGT, then SULF and Am4’CGT should exhibit complementary expression patterns. Analysis of RNA extracted from yellow and nonyellow regions of the petals of A. majus showed that SULF was preferentially expressed in the nonyellow region, whereas Am4’CGT was mainly expressed in the yellow region (Fig. 3D). The spatial restriction of Am4’CGT was confirmed by RNA in situ hybridization (Fig. 3E).

Overall expression of Am4’CGT was lower in petals of SULF compared with sulf-660 (Fig. 3F). 5’ RACE (rapid amplification of cDNA ends) on SULF genotypes revealed products for Am4’ CGT terminating at a range of positions, suggesting cleavage at multiple sites (fig. S8). No clear bands at the size expected for cleavage products were found in sulf. The lack of a single cleavage site in SULF genotypes is consistent with the SULF inverted duplication generating multiple sRNAs targeting Am4’CGT (Fig. 3B). To determine whether SULF alleles from the subspecies also varied in their ability to repress Am4’CGT, we introgressed SULF from A. m. pseudomajus (SULFp) or A. m. striatum (sulf) into an A. majus background with the same Am4’CGT target allele. Am4’CGT expression was reduced in both dorsal and ventral petals of SULFp compared with sulf (Fig. 3F). Thus, SULF acts by repressing transcript levels of the target Am4’CGT gene in A. m. pseudomajus but not in A. m. striatum.

If selection on inverted duplications is a common mechanism for establishing regulatory interactions, we might expect the genome to contain a large number of inverted duplications similar to SULF. Scanning the A. majus...
genome for inverted duplications with a similar adjusted folding energy to SULF revealed many such regions, some of which generated sRNAs (Fig. 4A). However, most of these sRNAs were >21 nucleotides (nt) long, unlike those generated by SULF (circled, Fig. 4B), which were ~21 nt. Moreover, the sRNA population generated by SULF was of relatively low complexity (ratio of the number of unique reads to total reads) because of the high abundance of a subset of sRNAs. Based on size and complexity, the profile of sRNAs generated by SULF was similar to that of conserved microRNA loci (orange spots, Fig. 4B). Given that the SULF hairpin is about five times long as a typical conserved microRNA hairpin, these findings suggest that SULF generates a functioning long regulatory hairpin RNA.

If only a subset of sRNAs generated by SULF are required to inhibit target gene activity, selection would not be able to maintain homology with the target gene Am4′CGT over the extended length observed (590 bp). This argument implies that SULF is of recent evolutionary origin, consistent with the phylogenetic analysis (Fig. 2B). With respect to its young age, SULF is similar to other inverted duplications with extended similarity to protein coding regions that encode sRNAs (14–17). Over evolutionary time, functional inverted duplications such as SULF might be lost, maintained, or become shorter microRNA hairpins (14, 15, 18–21). The deletions observed in both the left and right arms of the inverted repeat at SULF, relative to Am4′CGT (Fig. 2A), suggest that the process of size reduction may have already occurred to some extent.

Among the many documented cases of loci contributing to natural variation (22), several examples of small regulatory RNAs have been described (23–26). However, these examples involve changes in expression pattern of preexisting microRNAs or creation of new target sites, rather than de novo generation of a small regulatory RNA, as observed with SULF. The unusual nature of SULF may be a matter of chance or may reflect constraints on regulatory mechanisms (27).

For example, the biosynthetic pathway to yellow auroine pigment synthesis has fewer steps and has a more limited taxonomic distribution than the magenta anthocyanin pigment synthesis pathway (11, 28). Variation in transcription factors, such as ROS, may therefore not be available specifically to modulate yellow patterning. Inverted duplications that generate regulatory RNAs may thus provide a flexible mechanism, complementing that based on transcription factor or cis-regulatory variation (22), for modulating or creating novel expression patterns upon which natural selection may act to generate evolutionary change.

REFERENCES AND NOTES

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SUPPLEMENTARY MATERIALS
www.sciencemag.org/content/358/6365/925/suppl/DC1
Materials and Methods
Figs. 5 to 8
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References (30–59)
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How the snapdragon chooses its color

In some snapdragons, a yellow spot in a field of magenta shows the bee the best place to go. Flowers of a related subspecies are mainly yellow with magenta veins marking the target. Bradley et al. analyzed a locus that regulates the pattern of color. The locus contains an inverted gene duplication that encodes small RNAs that repress pigment biosynthesis. Analysis of flowers derived from a region of the Pyrenees where the subspecies coexist indicates that natural selection is operating upon the locus. Science, this issue p. 925