Plant materials and growth condition: The *Mimulus lewisii* inbred line LF10 was described in Owen and Bradshaw (2011) and Yuan et al. (2013). The inbred line SL9 was developed from another individual in the same population as the parent of LF10. The *guideless* mutant is from Owen and Bradshaw (2011). Greenhouse conditions are as described in Yuan et al. (2013).

Genome sequencing and assembly of SL9: To produce the reference SL9 genome assembly for the bulk segregant analysis, we generated 82 million 75-bp Illumina paired-end reads (12-fold average coverage) at the University of North Carolina High-Throughput Sequencing Facility (UNC-HTSF). We assembled these reads into 86,563 contigs (N50 = 2.3 kb) using CLC Genomics Workbench. These contigs were aligned to the 14 chromosomal-level super-scaffolds of *Mimulus guttatus* with the *nucmer* module from the MUMmer 3.0 package (Kurtz et al. 2004), assuming gene collinearity between *M. lewisii* and *M. guttatus*. A customized perl script “MUMmer_parser.pl” (available upon request) was written to connect the SL9 contigs into 14 “pseudoscaffolds” based on the MUMmer output.

Bulk segregant analysis of the *guideless* mutation by deep sequencing: An F2 population was produced from the cross between SL9 and a *guideless* mutant in the LF10 background, and 500 F2 individuals were grown to flowering. One hundred F2 segregants displaying the mutant phenotype were collected, and total genomic DNA was isolated from each of them using the BIO 101 System FastDNA kit (Qbiogene, Inc., Carlsbad CA). The concentration of each DNA sample was determined by using the PicoGreen dsDNA Quantitation Reagent (Invitrogen). The 100 DNA samples were then pooled together with equal representation from each segregant. A small-insert library (200-400 bp) was prepared for the pooled sample at UNC-HTSF, and 100-bp paired-end reads were generated by an Illumina HiSeq 2000.

The 277 million resulting reads (55-fold average coverage) were mapped to the 14 SL9 “pseudoscaffolds” with CLC Genomics Workbench, and 157,551 raw SNPs were detected. The *GUIDELESS* gene and tightly linked regions are expected to be homozygous for the LF10 genotype among all individuals displaying the mutant phenotype, which means that these regions are highly enriched in homozygous SNPs (Figure S2). The greater the distance from *GUIDELESS*, the more SL9 reads will be found. Upon reaching a point that is completely unlinked with *GUIDELESS*, the two genotypes (LF10 and SL9) will be randomly segregating in an expected proportion of 50%:50%.

The 157,551 raw SNPs were first filtered by depth of coverage. SNPs with >120-fold coverage were discarded because these regions are highly repetitive and, therefore, the reads were likely to be mapped incorrectly. The remaining 135,297 SNPs were then filtered by their tendency to cluster. The average SNP density between LF10 and SL9 is less than 0.002 (1 SNP every 500 bp); therefore, the highly clustered SNPs (3 or more SNPs in a 100-bp region) were likely to be caused by incorrect mapping. A total of 36,219 high quality SNPs were kept after filtering out clustered SNPs. The third step is to filter out heterozygous SNPs. SNPs with variant frequency less than 95% were considered as heterozygous. As a result, 3,450 high quality, homozygous SNPs were retained. The fourth step is to search for regions that are highly enriched in homozygous SNPs. The SL9 pseudoscaffolds were binned into 20-kb intervals, and the numbers of homozygous SNPs in each 20-kb interval were plotted in a bar graph (Figure 2A).

Two customized perl scripts, “SNP_filter.pl” and “HomoSNP_enrichment.pl” (available upon request), were written to automate the process of filtering SNPs and searching for homozygous SNP enrichment.

Plasmid construction and plant transformation: An RNAi plasmid was constructed with a 339-bp fragment from the third exon of *GUIDELESS*, essentially following the protocol described in Yuan et al. (2013). The primer pair *GUIDELESS_RNAi_F* and *GUIDELESS_RNAi_R* (Table S1) was used to amplify the 339-bp fragment. This fragment was BLASTed against the LF10 genome assembly with an E-value cutoff of 0.1 to ensure that no other genomic regions perfectly match this fragment for a contiguous block longer than 16 bp. The final plasmid construct was verified by sequencing and then transformed into *Agrobacterium tumefaciens* strain GV3101 for subsequent plant transformation, as described in Yuan et al. (2013).

Qualitative RT-PCR: Total RNA was isolated from root, stem, leaf, calyx and 6 stages of corolla development of LF10. RNA extraction and cDNA synthesis followed Yuan et al. (2013). The gene-specific primers *GUIDELESS_SP3F* and *GUIDELESS_SP3R* (Table S1) were used to amplify a 208-bp fragment of the third exon, to examine *GUIDELESS* expression in the wild-type LF10 across different tissue type and different stages of corolla development.
Gene-specific primers for the other seven MIXTA-like genes, \textit{MIMYBML1-7} (Table S1), were used to examine their expression at five different stages of corolla development (Figure S5). \textit{MIUBC} was used as a reference gene.

**Quantitative RT-PCR:** qRT-PCR was performed to quantify expression levels of \textit{GUIDELESS}, \textit{MIMYBML2}, \textit{MIMYBML6}, and \textit{MIMYBML7} in the 10-mm corolla of the wild-type LF10 and the four RNAi transgenic lines (RNAi-1, -4, -5, and -13). \textit{MIUBC} was used as a reference gene. The same gene-specific primers were used as qualitative RT-PCR. Three independent biological replicates of each line were analyzed, essentially following the procedure described in Yuan \textit{et al.} (2013). We determined amplification efficiencies for each primer pair using critical threshold values obtained from a dilution series (1:4, 1:20, 1:100, 1:500).

**Scanning electron microscopy:** Flower petal lobes and the nectar guides (the part of the ventral petal without the petal lobe) were dissected, fixed overnight in Formalin-Acetic-Alcohol (FAA) at 4°C, dehydrated for 30 min through a 50%, 60%, 70%, 95%, and 100% alcohol series. Samples were then critical-point dried, mounted, and sputter coated before being observed in a JEOL JSM-840A scanning electron microscope (University of Washington Biology Imaging Facility).

**Supporting References**
Yuan, Y. W., J. M. Sagawa, R. C. Young, B. J. Christensen, H. D. Bradshaw, Jr., 2013. Genetic dissection of a major anthocyanin QTL contributing to pollinator-mediated reproductive isolation between sister species of \textit{Mimulus}. Genetics doi:10.1534/genetics.112.146852