Loss of color pigmentation is maintained at high frequency in a monkey flower population

Loss of colour pigmentation is maintained at high frequency in a monkey flower population

Alex D. Twyford[1,2], Aaron M. Caola[2], Pratibha Choudhary[2], Ramesh Raina[2], Jannice Friedman[2]

[1] University of Edinburgh, Institute of Evolutionary Biology, West Mains Road, Edinburgh, EH9 3FL, UK

[2] Department of Biology, Syracuse University, 107 College Place, Syracuse, NY, 13244 USA

Author for correspondence: Alex D. Twyford

Tel: 0131 6508672

email: Alex.Twyford@ed.ac.uk

Natural History Miscellany

Online enhancements: Appendix

Keywords: anthocyanin, genetic variation, *Mimulus guttatus,* polymorphism

Running head: Colour polymorphism in *Mimulus*

Data archival location: Progeny segregation ratios are given in Table A2. Raw phenotypic data from the common garden growth trial, drought experiment, and UV experiment are given in Dryad DOI: doi:10.5061/dryad.17889**.**

Abstract

Colour polymorphisms have long been of evolutionary interest for their diverse roles including mate choice, predator avoidance, and pollinator attraction. While colour variation is often under strong selection, some taxa demonstrate unexpectedly high frequencies of presumed deleterious colour forms. Here we show a genetic variant underlying complete loss of anthocyanin pigmentation has risen to an unexpectedly high frequency of >0.2 in a natural population of the plant *Mimulus guttatus*. Decreased expression of *MYB5* transcription factor is associated with unpigmented morphs. While the allele was only found in heterozygote adults in the wild, suggesting negative selection, experiments were unable to demonstrate a fitness cost for unpigmented plants, suggesting a cryptic selection pressure in the wild. However, life history differences among morphs suggests unpigmented individuals benefit from later flowering and clonal growth. Overall, our study highlights the complex interplay of factors maintaining variation in nature, even for genes of major effect.

Introduction

The maintenance of polymorphisms at genes of major phenotypic effect has long interested evolutionary biologists. Before modern molecular biology, studying genetic variation in natural populations was limited to visible phenotypes with a known genetic basis, often controlled by one or a few loci. Early theoretical population geneticists used this variation to study the roles of selection and other forces such as genetic drift, gene flow and mutation in maintaining variation within and between populations (Fisher 1930; Haldane 1932; Wright 1931). Understanding the relative effect of these forces remains a central question in evolutionary biology (Delph and Kelly 2014; Mitchell-Olds et al. 2007). Studying major gene polymorphisms allows researchers to address whether phenotypic convergence involves repeatable genetic changes, whether adaptive mutations are more likely to occur in coding or regulatory regions, whether alleles are dominant or recessive, and assess the relative contributions of adaptive evolution, balancing selection, deleterious variation, and genetic drift in maintaining variation.

Many cases of major gene polymorphisms involve variation in colour polymorphism, which can be driven by natural or sexual selection and have profound effects on fitness (e.g. (Hoekstra et al. 2006; Reed et al. 2011; Rosenblum 2006;). A recent series of articles and discussion (Bolton et al. 2015; Bolton et al. 2016; Forsman et al 2008; Forsman 2016;Wennersten and Forsman 2012) illustrates that the direct and indirect effects of colour polymorphism on fitness can be complicated, and whether such polymorphism contributes to population persistence is contentious. Although these papers were focussed on animal examples, plants too show dramatic variation in colouration of vegetative and floral structures, and there are many notable cases where variation is present between closely

related species (Rausher 2008), among populations (Sobral et al. 2015), or show segregation within populations (Brown and Clegg 1984).

One of the major classes of plant pigments are anthocyanins, which are a conserved group of phenolic compounds responsible for the pink, red and blue colours produced by plants. Their conserved nature is also reflected by their conserved genetic basis; flowering plants share seven core enzymes of the anthocyanin biosynthetic pathway (Quattrocchio et al. 1993). Changes or loss of pigment can occur due to mutations in core structural genes encoding enzymes, or in the transcription factors regulating these genes (Ho and Smith 2016). These coloured compounds play diverse adaptive roles depending on the tissue where they are expressed. In flowers and fruits they may be involved in pollinator attraction and seed or fruit dispersal, while in vegetative tissues they may have protective roles in response to abiotic and biotic stressors, including UV radiation, drought, cold, herbivory, and pathogen defence (reviewed in Rausher 2008; Strauss and Whittall 2006). This tissue specific regulation is possible due to anthocyanin biosynthetic pathway gene regulation at the transcriptional level by the MYB-bHLH-WD repeat complex (Davies et al. 2012; Koes et al. 2005), and studies show that concentrations of floral and leaf anthocyanins and flavonols are not always correlated (Berardi et al. 2016; del Valle et al. 2015).

Studies in the genus *Mimulus* (monkey flowers) have been informative for understanding plant pigmentation (Twyford et al. 2015; Wu et al. 2007). For example, in their now classic study, Bradshaw and Schemske (2003) show that the shift from bee to hummingbird pollination involved floral pigmentation changes caused by a few genes of major effect. In general, the ~170 monkey flower species show numerous flower colour transitions among related species, and the genetic basis of pigmentation in the group has been well studied (e.g. Cooley et al. 2011; Streisfeld and Rausher 2009; Yuan et al. 2016). In a previous common garden experiment of species-wide collections of *M. guttatus*, we noticed a

single atypical plant from California lacking red spotting on flowers and having bright green leaves, in contrast to the red spotted flowers and red tinged leaves typical of the species (fig. 1). While there are many examples of anthocyanin loss from floral tissue, whole-plant loss of anthocyanin is uncommon in nature (though see Warren and Mackenzie 2001; Wu et al. 2013). Our expectation was that this anthocyanin-less phenotype should be rare in the wild as anthocyanin is present in plants from across the range of *M. guttatus* (and indeed widely found across all species of flowering plants, Lawrence et al. 1939), and anthocyanin plays a diverse and important adaptive role. Therefore a complete loss of anthocyanin is unexpected and an obvious target for removal via purifying selection.

In this study, we pursue our observation of a naturally occurring unpigmented phenotype of *M. guttatus*, and use this as an opportunity to investigate the genetics and maintenance of intrapopulation phenotypic variation and the role of anthocyanin for plant performance. We address the following questions: (1) What is the genetic basis of this variation? (2) What is the frequency of this variant in the wild? (3) What are the fitness effects of this phenotype? We address these questions by analysing phenotypic segregation in experimental crosses and wild collected seed families, by measuring fitness effects in a common garden study and under experimental conditions where plants are exposed to relevant environmental stresses, and through gene expression analysis. Our results show the complexities of how an intriguing major polymorphism underlying an important phenotypic trait can be maintained in a natural population.

Methods

Population description and sampling scheme

In July 2013 we visited the study population in Hume, Sequoia National Forest, California, at N36°46'58.02", W118°53'55.56", and 5330ft above sea level. *Mimulus guttatus* at the site belong to the perennial ecotype. We estimated the census population size at approximately 300 flowering individuals, and no other known *M. guttatus* plants grow within a 3km range. We phenotyped plants in the field for anthocyanin, by scoring for spotting on the flowers (if present) and the visual presence of anthocyanin in the petiole as a proxy for vegetative anthocyanin. We collected open pollinated seed capsules for segregation analysis, selecting 27 maternal parents each at least 1m apart to reduce the likelihood of collecting clonal individuals.

Phenotypic description and estimates of allele frequencies

We grew seed from the 27 field-collected seed families in the greenhouse at Syracuse University, with an average of 21 (range 6-32) seedlings per family raised to flowering. Seeds were planted in plug trays with Fafard 4P potting mix, stratified at 4°C in the dark for one week, and grown at 21°C and 16-hour photoperiod (16L/8D) in a greenhouse. Flowers and leaves were scored for the presence or absence of pigmentation. We used segregation ratios of these field collected seed families to infer the maternal genotype, and to estimate the frequency of the unpigmented allele in nature (described below).

We confirmed that plants are either absent in anthocyanin, or produce undetectable levels in flowers and in leaves, by extracting anthocyanin with methanol-HCl from 4 pigmented and 4 unpigmented plants, and performing thin layer chromatography in a solvent of BAW, BuHCl and 1% HCl (Harborne 1998). Pigments were identified based on published retention factors (R_f) in this solvent.

We tested the genetic basis of the anthocyanin phenotype by observing segregation in experimental crosses. We produced two second-generation inbred individuals from single-seed descent, selected for producing progeny that did not segregate for anthocyanin to confirm that they were homozygous. These alternate homozygous parents were inter-crossed to produce an F1, with anthocyanin scored in the subsequent F2s. We raised plants to flowering in the greenhouse, and then tested whether the presence/absence of anthocyanin differed from the 3:1 ratio expected from segregation at a single Mendelian locus with a chi-squared test. We determined whether unpigmented plants are plastic for anthocyanin production, by exposing them to a high light intensity stressful environment that typically induces anthocyanin (Albert et al. 2009). We recorded the presence or absence of anthocyanin after one week of exposure to natural UV-light conditions and drought on the roof of the Life Science Complex at Syracuse University, in a set of 48 unpigmented and 48 wild type F2 plants.

RNA extraction and reverse transcription (RT)-PCR

The class R2R3 type of *MYB*s are regulators of anthocyanin biosynthesis in a wide range of plant species (Liu et al. 2015). Recently, five R2R3 types of *MYB* genes were identified in *M. guttatus* (Cooley et al. 2011) and thus are good candidates for regulators of anthocyanin. To test if any of these *MYB* genes regulate anthocyanin accumulation, we examined the transcript levels of *MgMYB1-5* in the leaves and floral buds of *AA*, *Aa*, *aa* plants. We collected two-week-old leaves and unopened floral buds from four plants of each genotype and pooled them for RNA isolation. We isolated total RNA using TRIzol reagent (Invitrogen, USA) as per manufacturer's instructions, and determined RNA concentration on agarose gel and by spectrophotometer (Bio-Rad, USA). We retrieved *MgMYB1-MgMYB5*

gene sequences from http://www.phytozome.net, and designed appropriate primers. Details of primer sequences, cDNA synthesis, and RT-PCR reactions are given in the appendix. We used *Mimulus guttatus UBIQUITIN-CONJUGATING ENZYME* (*UBC*) gene as a normalization control (Yuan et al. 2014). As a positive control for PCR, we used genomic DNA isolated using Edward's protocol (Edward et al. 1991).

Measures of fitness

We tested whether the absence of anthocyanin affects vigour, pollination and seed set between genotypic classes when grown outside in an experimental field. Initially, we identified 10 unrelated homozygous wildtype and 7 unrelated homozygous mutant plants by selfing plants from the segregating families of field-collected plants, and retaining only those parents that did not produce any segregating progeny. These plants then served as the parents for our crossing scheme. Plants were crossed in a round-robin design to generate outcrossed individuals of three genotypic classes (outcrossed pigmented [6 crosses], outcrossed unpigmented [6 crosses], pigmented x unpigmented [6 crosses]). In 2014, a total of 116 individuals, consisting of 6-7 full-sib individuals from each of these 18 crosses, were planted in a randomised block design in an experimental field site at Syracuse University. We measured a suite of vegetative and flowering traits at three-week intervals: plant height and width, the number of stolons, and number of flowering branches. In addition, we recorded leaf length (three weeks post-transplant), the date of first flower, and ranked the plants for senescence on a 5-point scale at the end of the season. Flowers were available to be visited by pollinators, and we collected at least 2 seed capsules from each plant prior to dehiscing, and counted the number of seeds per capsule on a random subset of 40 plants. We provided plants

with daily supplemental water, until all plants were finishing flowering. The following spring we assessed over-winter survival by recording any plants with signs of regrowth.

We conducted two separate growth chamber experiments, where we investigated the effect of exposure to UV light, and the effect of drought conditions. For each experiment, we used a total of 12 crosses (four of each genotype) with 8 full-sibs per family. In both experiments, 192 plants were grown for 10 weeks in a growth chamber with 16:8-hr day:night at 21:18°C. In the UV experiment, half the plants were assigned to a control treatment with regular light (PAR=250, UV=10 $\mu W/cm^2$) and the other half were assigned to a UV treatment with supplemental UV light (PAR=258, UV= 2360 $\mu W/cm^2$). In the second experiment, half the plants were watered with our regular regimen of soaking flats for one hour daily, while the other plants were watered every four days. For both experiments, we measured plants for germination timing, and a suite of growth, flowering and senescence traits at regular intervals.

We analysed the fitness experiments with REML general linear models in SAS (mixed procedure, release 9.2, SAS Institute Inc. 2015) Within each experiment, dependent variables were standardized to a mean of zero and standard deviation of one (to facilitate comparisons between experiments). For the field experiments, analyses included the effect of genotype as a fixed categorical factor, and block and family nested with genotype as random factors. The significance of family was assessed using a log-likelihood ratio test, and a X^2 test statistic (Littell et al., 1996). For the UV and drought experiments, we used similar models, and also included the fixed effect of treatment and its interaction with genotype. We analysed pairwise significant fixed effects with Tukey's method, which adjusts the Type I error rate for multiple testing.

Results

Phenotypic observations

Our greenhouse observations made on all 568 individuals from 27 wild collected seed families used across experiments, revealed two discrete classes of pigmentation, with individuals either having anthocyanin, or plants being entirely unpigmented (fig. 1). Thin-layer chromatographic analysis of floral and leaf extracts from four pigmented and four unpigmented plants confirmed that unpigmented plants do not produce detectable levels of pigment with a retention factor (R_f) in the range expected of anthocyanin, in contrast to the pigmented plants. We observed that 48 unpigmented greenhouse-grown plants, which were then exposed to natural UV-light conditions and drought on the Syracuse University rooftop, still did not produce visible anthocyanin (Results not shown). These results show the unpigmented phenotype is genetic and can reliably be scored as a simple binary trait under glasshouse conditions.

Genetic basis

We used segregation in an F2 family derived from a cross between an inbred pigmented, and inbred unpigmented plant, to investigate the genetic basis of the phenotype. The F2 progeny segregated in a ratio of 108:29 pigmented:unpigmented, which did not significantly differ from the 3:1 ratio expected from simple Mendelian segregation (χ^2=1.073, P=0.3). There was perfect co-segregation between floral and vegetative anthocyanins in the progeny, and in wild collected families (below), suggesting loss of anthocyanin in flowers and vegetative tissue is controlled by a single locus. As three quarters of F2s, and all F1 plants, produce anthocyanin, the presence of anthocyanin is dominant. As

such the unpigmented phenotype is controlled by a single allele *a,* which is recessive to the wildtype allele *A*.

Transcription of MYB1-5 genes

We examined transcript levels of *MYB1-5* in the leaves and floral buds of *AA*, *Aa*, *aa* plants to test for differences among genotypes. Only *MYB4* and *MYB5* transcripts were detected in the leaves and floral buds of *AA*, *Aa*, and *aa* plants, consistent with the previous report of not detecting transcripts of *MYB1-3* in the floral buds of an inbred line (IM767) of *M. guttatus* (Yuan et al. 2014). Level of *MYB4* transcript was similar in the leaves and floral buds of all three genotypes. Only *MYB5* transcript was differentially expressed in the three genotypes, for both leaves and floral buds (fig. 2A). Transcript levels were highest in *AA* plants and lowest in *aa* plants (fig. 2A). This correlates with the visible level of anthocyanin accumulation in these plants – highest in the *AA* and lowest in *aa* plants (fig. 1). Taken together, our results suggest that MYB5 positively regulates anthocyanin accumulation in *M. guttatus,* and decreased expression is associated with the anthocyanin deficient phenotype.

Allele frequencies in the wild

We estimated the frequency of the anthocyanin allele in the wild using information from the phenotypic class of the parent scored in nature, and the progeny segregation ratios. All plants phenotyped in the wild produced anthocyanin and thus are genotype *AA* or *Aa*. Eleven of the 27 seed families (40%) raised from these plants subsequently segregated for pigmentation (mean proportion unpigmented: 0.23, range: 0.06-0.36, see Table A2), and thus the maternal parent has genotype *Aa*. The remaining sixteen families did not segregate for

anthocyanin production. These may have maternal parents with genotype *AA*, or genotype *Aa* where no homozygous recessive progeny are present due to the preponderance of the dominant *A* allele in the pollen pool, or due to small sample sizes for some families. Assuming these parents with no segregating offspring are *AA*, gives a conservative lower estimate of 0.2 for the *a* allele (ie. 11 *Aa* and 16 *AA*, frequency of *a* is 11/54). To give an upper estimate on the frequency of *a*, if all non-segregating plants were actually heterozygous (i.e. 27 *Aa*), the frequency of *a* would be 0.5.

Measures of fitness

In our experimental field common garden with 116 outcrossed individuals of known genotype, the genotypes differed in some growth measures. They did not differ in leaf length (three weeks after transplant) or seed set (Table 1). Unpigmented plants flowered later (mean days to flower: *AA* 51.6 ± 0.9; *Aa* 53.02 ± 1.03; *aa* 61.0 ± 1.05) and made fewer flowering branches (mean branches: *AA* 5.0 ± 0.49; *Aa* 5.3 ± 0.5; *aa* 2.6 ± 0.43), but made significantly more stolons (mean stolons: *AA* 1.6 ± 0.26; *Aa* 2.9 ± 0.32; *aa* 5.8 ± 0.27) (fig. 3). For all traits, except stolon production, the *AA* and *Aa* genotypes did not differ significantly from one another. Nonetheless, when we calculate gene action of each allele at the single locus, all traits show either partial dominance or overdominance (Table 1).

In the growth chamber experiments with either supplementary UV light or drought treatments, we found that similar to the field experiment, genotypes differed significantly for most traits, driven by differences between the *aa* genotype and the other two genotypes (Table 1). We expected *aa* genotypes to be most affected by the stressors, however this was not borne out. Within an experiment the genotypes generally responded similarly to the stressors, for example by flowering significantly later under UV and significantly earlier

under drought (fig. 3A). In both experiments, there was a significant genotype x treatment interaction for stolons. Plants of *aa* genotype increased their stolon production in both UV and drought, while the other genotypes either decreased stolons or did not change significantly from control (fig. 3B).

Discussion

We have described the genetic basis, frequency, and fitness effects of a segregating colour polymorphism maintained in a natural population. The absence of anthocyanin in some plants of the Hume population of *M. guttatus* is controlled by a single recessive allele, which negatively affects expression of the *MYB5* transcription factor. The causal allele is present at a surprisingly high frequency of >0.2 in this population. Our experiments did not identify a cost of this allele on attractiveness to pollinators or on survival in a field environment, and plants with this phenotype performed well in response to environmental stressors including high UV light and drought. One clue to how this variant is maintained may come from the close association between the recessive phenotype and an alternative life history strategy—unpigmented plants consistently flowered later and invested more in stolons. We consider below how these results improve our understanding of the maintenance of phenotypic variation.

The unpigmented *M. guttatus* phenotype represents a natural variant where anthocyanin is not produced in any tissue of the plant, and crosses revealed floral and vegetative anthocyanin always cosegregate. This is unusual as most plants with mutations for unpigmented flower variants still produce some anthocyanin in stems and leaves (Warren and Mackenzie 2001), or the loss of anthocyanin occurs independently in flowers and vegetative tissues (e.g. Dick et al. 2011). However, estimating the occurrence of complete loss of

anthocyanin phenotypes is challenging, as many studies only report the presence of pigment in flowers and not in vegetative tissues, or do not discriminate between reduced pigment levels and the absence of pigmentation.

Selection pressures and the maintenance of variation

The maintenance of major gene polymorphisms within a single population are intriguing, especially cases such as this where a presumably deleterious allele has risen to a relatively high frequency (>0.2). This frequency is substantially higher than many other loss-of anthocyanin mutations that segregate within populations (e.g. *Mimulus lewisii*, 0.03, Wu et al. 2013; *Ipomoea purpurea*, 0.005, Coberly and Rausher 2003). To date, most empirical studies in animals suggest such polymorphisms are maintained by negative frequency-dependent selection, mediated by apostatic or sexual selection (Gray and McKinnon 2007). We cannot see a scenario in which the unpigmented and pigmented plants experience a selective advantage when rare, and so we do not think this is the mechanism responsible for its maintenance. We can also cast doubt on some other possible explanations, such as genetic drift, mutation-selection balance, or heterozygote advantage. Random genetic drift could be causal, and has been implicated in segregating colour polymorphisms in northern leopard frogs (Hoffman et al. 2006) and the candy-stripe spider (Oxford 2005). In the latter example, the rare morph occurs at low frequencies (~0.05-0.3) in small populations, and the authors suggest that selection is weak and drift dominates. Although 'intermittent drift' (Oxford and Shaw 1986) may be involved in this *M. guttatus* population, the correlated phenotypic effects of anthocyanin on other traits, discussed below, suggests genetic drift is unlikely the main mechanism. We also believe mutation-selection balance is unlikely, as we did not find complementary gene action when performing crosses amongst different families of

unpigmented plants (i.e. offspring were unpigmented, suggesting the causal mutation is in a single gene). It seems improbable that a mutation keeps arising in the same gene within this population. There is some indication of heterozygote advantage, as we find overdominance for some traits (height [not shown], leaf length, flowering stems: Table 1), but its unclear whether these traits result in higher fitness. Thus we are reluctant to suggest that the polymorphism is maintained by overdominance at this single locus in a single population ('simple overdominance': Delph and Kelly 2014), especially given how rare this phenomenon is in nature (though see Johnston et al. 2013; Tuttle 2003).

A probable explanation for the maintenance of the polymorphism is that selection is acting on suites of traits that include the pigment phenotype, rather than the pigment trait itself. Our fitness and growth experiments show that homozygote recessives (*aa*) make more stolons (clonal growth), flower later and for longer. Overall, the *aa* genotype has a more 'perennial' life history strategy, investing more in vegetative growth with delayed flowering (Friedman et al. 2015). This finding is not unique to *Mimulus*, indeed many organisms demonstrating major polymorphism exhibit true alternative life history strategies (Silva et al. 2015; Tuttle 2003). As such, selection may be acting on life history traits, with a more clonal phenotype being advantageous in this permanently wet site that facilitates late-season flowering and persistence via clonal reproduction. This scenario would entail pleiotropic gene effects or physical linkage between life history traits and the pigmentation gene (discussed below). Thus, it is possible that clonality may be involved in the maintenance of the unpigmented plants, particularly if the population was founded by a small number of individuals. To assess whether selection is acting through stolon production would require measuring lifetime fitness in the field for this perennial plant, and assessing fitness components through survival, stolons and seed production.

We also cannot exclude the possibility that the maintenance of the phenotype is due to complex selection pressures that occur in the wild, including fine-scale spatial and temporal differences in selection, potentially in conjunction with selection on cryptic trait variation. This seems plausible as even systems with intensely studied colour polymorphisms often fail to locate the target of selection (reviewed in Gray and McKinnon 2007). This issue may be particularly problematic with pigments such as anthocyanins that play extremely diverse functions, and where fitness in an experimental field may fail to capture components of selection in the wild (Waser and Price, 1981). We found unpigmented plants had equivalent seed set to pigmented plants in a field setting, and if this was also the case in the native environment, it would suggest the generalist pollinators that visit *M. guttatus* do not discriminate against plants with subtle shifts in flower colour from yellow with red spots to unspotted flowers. Similarly, our growth experiments showed unpigmented plants grow well under field conditions, as well as in growth chamber experiments with elevated UV light and drought conditions. These findings are consistent with *Arabidopsis* anthocyanin mutants that had equivalent fitness to wildtype plants under stressful conditions (von Wettberg et al. 2010), although *Petunia* mutants defiicient in *F3'H* grew more slowly under UVB than wildtype plants (Ryan et al. 2002). One obvious pressure we did not directly investigate is herbivory. Anthocyanin strongly affects the preference of many herbivores (Strauss and Whittall 2006), and herbivory tests with slugs and Lepidoptera show a preference for non-pigmented over pigmented flowers in wild radishes (*Raphanus sativus*) (Irwin et al. 2003). We have also not investigated whether loss of anthocyanin has benefits in terms of resource expenditure, particularly in limiting environments.

Genetic basis

Loss of anthocyanin can occur due to mutations in structural genes, or in transcription factors that regulate the expression of structural genes. While loss of anthocyanin from all plant tissues is usually attributed to mutations in structural genes, *MYB5* appears to be a candidate transcription factor that regulates anthocyanin expression across the plant. Our gene expression analysis found *MYB5* was the only differentially expressed candidate transcription factor consistent with the observed phenotypes. The role of this transcription factor is supported by Yuan et al. (2014), who showed *MYB5* is the only anthocyanin activating *R2R3-MYB* transcription factor (sensu Cooley et al. 2011) expressed in the corolla of *M. guttatus*. In the related *Mimulus lewisii*, mutations in *MlWD40a* (part of the *MYB-bHLH-WD40* regulatory complex) affected anthocyanin accumulation in both flowers and stems, while mutations in *R2R3-MYB*s, including those most closely related to *MgMYB5* only affected floral anthocyanin. Similarly, in *M. aurantiacus*, *MaMYB2* (most closely related to *MgMYB5*) is necessary for floral anthocyanin pigmentation (Streisfeld et al. 2013; Streisfeld & Rausher 2009). Finally, Lowry et al. (2012) show that variation amongst populations of *M. guttatus* in leaf and floral anthocyanin can be ascribed to an *R2R3-MYB* cluster (but not including *MYB5*), however their study deals with differences in anthocyanin intensity and pattern, and not presence/absence. While we have identified the likely involvement of *MYB5*, further work will be required to prove its role as the casual gene, and to understand the molecular mechanism underlying the phenotype. This could be tested by determining the expression of genes in families segregating for the anthocyanin phenotype, or by using recently developed transformational protocols for *M. guttatus* (Preston et al. 2014).

Our hypothesis that natural selection is not acting directly on anthocyanin genes, but on correlated life history traits, raises the prospect of physical linkage between (a regulator of) *MYB5* and life history traits. While there are many candidate genes underlying the perennial strategy in *M. guttatus*, the most obvious is the large *DIV1* inversion that has a

major phenotypic effect (Lowry and Willis 2010). Although this inversion contains a tandem *MYB* array responsible for some anthocyanin phenotypes in *M. guttatus* (Lowry et al. 2012), it is not the location of the *MYB5* locus that has reduced expression. *MYB5* is on Linkage Group 12, at position 6,076,089 (Migut.L00458 phytozome.jgi.doe.gov). There are not many candidate genes in this region, but a gene showing homology to *PROTEIN SUPPRESSOR OF PHYA-105* (*SPA1*) is located about 1MB away at 7,216,182 (Migut.L00551). *SPA1* is known to negatively regulate anthocyanin accumulation, plant size, and flowering in *Arabidopsis*, especially in short days (Ishikawa et al. 2006). Hypothetically, an accumulation of *SPA1* in *aa* plants, might explain its decreased anthocyanin accumulation, bigger vegetative growth, and delayed flowering phenotypes. Future work will be necessary to test these hypotheses.

Conclusion

The maintenance of multiple variants within a population provides a rare opportunity to explore allele dynamics and phenotypic variation within a shared environmental context. Although we have been unable to identify the ecological mechanisms favouring the colour polymorphism in this population, the high allele frequency suggests that there is selection for either the colour variant, or it is favoured via pleiotropic effects or linkage disequilibrium with other traits under selection. We suggest that this provides an opportunity to examine the interconnections between evolutionary processes such as different forms of selection and drift, and to link ecological mechanisms favouring polymorphisms with their genetic basis.

Acknowledgements

This work was supported by Syracuse University, National Science Foundation grant (DEB-1354259) to JF, and Heredity Field Work Grant to ADT. ADT is supported by Natural Environment Research Council Fellowship NE/L011336/1. We thank John Paul for information about the Hume population, and Abrar Aljibroury, Anna Bjarvin and Matthew Rubin for help with the experiments. ADT and JF wrote the manuscript, and all authors approved the final version. No competing financial interests to report.

Appendix

RNA isolation and PCR conditions

We isolated total RNA using TRIzol reagent (Invitrogen, USA) per manufacturer's instructions, and determined RNA concentration on agarose gel and by spectrophotometer (Bio-Rad, USA). We used 5µg of total RNA for cDNA synthesis in a 20µl reaction using RevertAid reverse transcriptase (Thermo-Fisher Scientific, USA). We further diluted cDNA samples with 20µl sterile water. For each RT-PCR reaction, we combined 1-2µl of cDNA, 1.5µl of 10X Taq Pol buffer (BioBasic, USA), 1.5µl of 20 mM MgSO4, 1.5µl of 2.5 mM dNTPs mix (BioBasic, USA), 0.4µl of 10µM forward and reverse primers, one unit of TaqPol enzyme (BioBasic, USA), and sterile water to a final volume of 15µl. We performed PCR reactions as follows: first step at 95˚C for 5 min, followed by cycles (listed below) of 95˚C for 30 sec, annealing temperature based on primers for 30 sec, and 72˚C for 30 sec to 60 sec.

Table A1: Primer sequences and PCR conditions used for gene expression analysis of *R2R3-MYB* genes in *M. guttatus*

Primer Name	5'-3' primer sequence	Cycle number	Amplicon length	Reference
MYB1-FP	5’-GACGAATGCATTAAATGGTGGGC-3’	45	~ 200 bp	This study
MYB1-RP	5’-CCCAATCATCGAAATCTAGGG-3’			
MYB2-RP	5’-GCGATGAAGTAATAGTAACGACG-3’	45	~ 180 bp	This study
MYB2-FP	5’-CGGCGGAAATTCTGCATCATC-3’			
MYB3-FP	5’-GTAGCAGTACTGCCACCTGCTAC-3’	45	~250 bp	This study
MYB3-RP	5’-GGTCCAGTGAATGGGTAAGCCTC-3’			
MYB4-RP	5'-GGAATCGCCAAATGAAATCGCG-3’	40	~180 bp	This study
MYB4-FP	5’-GTCCAAAAGTCCCAAATGCCC-3’			
MYB5-RP	5’-GCTTGAAACTACTACGACGGAAG-3’	35-37	~190 bp	This study
MYB5-FP	5’-CGTTGAATGTGAGAAGCTCCC-3’			
UBC-RP	5’-GGCTTGGACCCTGCAATCAG-3’	25	~200 bp	Yuan et al. 2014
UBC-FP	5’-CTTGGGCATGGCAGCAAGTC-3’			

Table A2: Segregation of anthocyanin pigmentation in 27 wild-collected seed families of *Mimulus guttatus* collected in Hume, California.

Family	Total	Unpigmented	Pigmented	Proportion
1	31	3	28	0.097
2	32	2	30	0.063
3	17	3	14	0.176
4	31	5	26	0.161
5	30	12	18	0.4
6	25	9	16	0.36
7	26	0	26	0
8	29	0	29	0
9	26	0	26	0
10	27	0	27	0
11	25	0	25	0
12	29	0	29	0
13	17	5	12	0.294
14	13	0	13	0
15	28	8	20	0.286
16	17	0	17	0
17	19	0	19	0
18	21	0	21	0
19	24	2	22	0.083
20	12	0	12	0
21	12	0	12	0
22	6	3	3	0.5
23	16	0	16	0
24	13	0	13	0
25	7	0	7	0
26	20	3	17	0.15
27	15	0	15	0
Total	568	55	513	0.097

Literature Cited

Albert, N. W., D. H. Lewis, H. Zhang, L. J. Irving, P. E. Jameson, and K. M. Davies. 2009. Light-induced vegetative anthocyanin pigmentation in *Petunia*. J. Exp. Bot. 60:2191-2202.

Berardi, A. E., S. B. Hildreth, R. F. Helm, B. S. Winkel, and S. D. Smith. 2016. Evolutionary correlations in flavonoid production across flowers and leaves in the Iochrominae (Solanaceae). Phytochemistry 130:119-127.

Bolton, P. E., L. A. Rollins, and S. C. Griffith. 2015. The danger within: the role of genetic, behavioural and ecological factors in population persistence of colour polymorphic species. Mol. Ecol. 24:2907-2915.

Bolton, P. E., L. A. Rollins, and S. C. Griffith. 2016. Colour polymorphism is likely to be disadvantageous to some populations and species due to genetic architecture and morph interactions. Mol. Ecol. 25:2713-2718.

Bradshaw, H. D. and D. W. Schemske. 2003. Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. Nature 426:176-178.

Brown, B. A. and M. T. Clegg. 1984. Influence of flower color polymorphism on genetic transmission in a natural population of the common morning glory*, Ipomoea purpurea*. Evolution 38:796-803.

Coberly, L. C. and M. D. Rausher. 2003. Analysis of a chalcone synthase mutant in *Ipomoea purpurea* reveals a novel function for flavonoids: amelioration of heat stress. Mol. Ecol. 12:1113-1124.

Cooley, Arielle M., Jennifer L. Modliszewski, Megan L. Rommel, and John H. Willis. 2011. Gene duplication in *Mimulus* underlies parallel floral evolution via independent trans-regulatory changes. Curr. Biol. 21:700-704.

Davies, K. M., N. W. Albert, and K. E. Schwinn. 2012. From landing lights to mimicry: the molecular regulation of flower colouration and mechanisms for pigmentation patterning. Funct. Plant Biol. 39:619-638.

del Valle, J. C., M. L. Buide, I. Casimiro-Soriguer, J. B. Whittall, and E. Narbona. 2015. On flavonoid accumulation in different plant parts: variation patterns among individuals and populations in the shore campion (*Silene littorea*). Front Plant Sci 6:939.

Delph, L. F. and J. K. Kelly. 2014. On the importance of balancing selection in plants. New Phytol. 201:45-56.

Dick, C. A., J. Buenrostro, T. Butler, M. L. Carlson, D. J. Kliebenstein, and J. B. Whittall. 2011. Arctic mustard flower color polymorphism controlled by petal-specific downregulation at the threshold of the anthocyanin biosynthetic pathway. PLoS ONE 6:e18230.

Edward, K., C. Johnstone, and C. Thompson. 1991. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. 19:1349.

Fisher, R., A. 1930. The Genetical Theory of Natural Selection. Clarendon Press, Oxford.

Forsman, A. 2016. Is colour polymorphism advantageous to populations and species? Mol. Ecol. 25:2693-2698.

Forsman, A., J. Ahnesjö, S. Caesar, and M. Karlsson. 2008. A model of ecological and evolutionary consequences of color polymorphism. Ecology 89:34-40.

Friedman, J., A. D. Twyford, J. H. Willis, and B. K. Blackman. 2015. The extent and genetic basis of phenotypic divergence in life history traits in *Mimulus guttatus*. Mol. Ecol. 24:111-122.

Gray, S. M. and J. S. McKinnon. 2007. Linking color polymorphism maintenance and speciation. Trends Ecol. Evol. 22:71-79.

Haldane, J. B. S. 1932. The Causes of Evolution. Princeton University Press, USA

Harborne, A. 1998. Phytochemical methods a guide to modern techniques of plant analysis. Springer Science & Business Media.

Ho, W.W. and S. D. Smith, S.D. 2016. Molecular evolution of anthocyanin pigmentation genes following losses of flower color. BMC Evol Biol. 16:98.

Hoekstra, H. E., R. J. Hirschmann, R. A. Bundey, P. A. Insel, and J. P. Crossland. 2006. A single amino acid mutation contributes to adaptive beach mouse color pattern. Science 313:101-104.

Hoffman, E. A., F. W. Schueler, A. G. Jones, and M. S. Blouin. 2006. An analysis of selection on a colour polymorphism in the northern leopard frog. Mol. Ecol. 15:2627-2641.

Irwin, R. E., S. Y. Strauss, S. Storz, A. Emerson, and G. Guibert. 2003. The role of herbivores in the maintenance of a flower color polymorphism in wild radish. Ecology 84:1733-1743.

Ishikawa, M., T. Kiba, and N.-H. Chua. 2006. The *Arabidopsis SPA1* gene is required for circadian clock function and photoperiodic flowering. The Plant Journal 46:736-746.

Johnston, S. E., J. Gratten, C. Berenos, J. G. Pilkington, T. H. Clutton-Brock, J. M. Pemberton, and J. Slate. 2013. Life history trade-offs at a single locus maintain sexually selected genetic variation. Nature 502: 93-95.

Koes, R., W. Verweij, and F. Quattrocchio. 2005. Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. Trends Plant Sci. 10:236-242.

Lawrence, W. J. C., J. R. Price, G. M. Robinson, and R. Robinson. 1939. The distribution of anthocyanins in flowers, fruits and leaves. Phil. Trans. R. Soc. B 230: 149-178.

Littell, R. C., G. A. Milliken, W. W. Stroup, R. D. Wolfinger. 1996. SAS system for mixed models. SAS Institute Inc: Cary, North Carolina.

Liu, J., A. Osbourn, and P. Ma. 2015. MYB transcription factors as regulators of phenylpropanoid metabolism in plants. Mol Plant 8:689-708.

Lowry, D. B., C. C. Sheng, J. R. Lasky, and J. H. Willis. 2012. Five anthocyanin polymorphisms are associated with an R2R3-MYB cluster in *Mimulus guttatus* (Phrymaceae). Am. J. Bot. 99:82-91.

Lowry, D. B. and J. H. Willis. 2010. A widespread chromosomal inversion polymorphism contributes to a major life-history transition, local adaptation, and reproductive isolation. PLoS Biol. 8:e1000500.

Mitchell-Olds, T., J. H. Willis, and D. B. Goldstein. 2007. Which evolutionary processes influence natural genetic variation for phenotypic traits? Nat Rev Genet 8:845-856.

Oxford, G. and M. Shaw. 1986. Long-term variation in colour-morph frequencies in the spider *Enoplognatha ovata* (Clerck)(Araneae: Theridiidae): natural selection, migration and intermittent drift. Biol. J. Linnean Soc. 27:225-249.

Oxford, G. S. 2005. Genetic drift within a protected polymorphism: enigmatic variation in color-morph frequencies in the candy-stripe spider, *Enoplognatha ovata*. Evolution 59:2170-2184.

Preston, J. C., L. L. Barnett, M. A. Kost, N. J. Oborny, and L. C. Hileman. 2014. Optimization of virus-induced gene silencing to facilitate evo-devo studies in the emerging model species *Mimulus guttatus* (Phrymaceae). Ann. Mo. Bot. Gard. 99:301-312.

Quattrocchio, F., J. F. Wing, H. T. Leppen, J. N. Mol, and R. E. Koes. 1993. Regulatory genes controlling anthocyanin pigmentation are functionally conserved among plant species and have distinct sets of target genes. Plant Cell 5:1497-1512.

Rausher, M. 2008. Evolutionary transitions in floral color. Int. J. Plant Sci. 169:7-21.

Reed, R. D., R. Papa, A. Martin, H. M. Hines, B. A. Counterman, C. Pardo-Diaz, C. D. Jiggins, N. L. Chamberlain, M. R. Kronforst, R. Chen, G. Halder, H. F. Nijhout, and W. O. McMillan. 2011. Optix drives the repeated convergent evolution of butterfly wing pattern mimicry. Science 333:1137-1141.

Rosenblum, E. B. 2006. Convergent evolution and divergent selection: lizards at the white sands ecotone. Am. Nat. 167:1-15.

Ryan, K. G., Swinny, E. E., Markham, K. R., Winefield, C., 2002. Flavonoid gene expression and UV photoprotection in transgenic and mutant *Petunia* leaves. Phytochemistry. 59: 23-32.

Silva, S. E., A. S. Rodrigues, E. Marabuto, S. Yurtsever, P. A. Borges, J. A. Quartau, O. S. Paulo, and S. G. Seabra. 2015. Differential survival and reproduction in colour forms of *Philaenus spumarius* give new insights to the study of its balanced polymorphism. Ecol. Entomol. 40:759-766.

Sobral, M., T. Veiga, P. Domínguez, J. A. Guitián, P. Guitián, and J. M. Guitián. 2015. Selective pressures explain differences in flower color among *Gentiana lutea* populations. PLoS ONE 10:e0132522.

Strauss, S. Y. and J. B. Whittall. 2006. Non-pollinator agents of selection on floral traits. Pp. 120-138 *in* L. D. Harder and S. C. Barrett, eds. *Ecology and evolution of flowers*, Oxford University Press.

Streisfeld, M. A. and M. D. Rausher. 2009. Altered trans-regulatory control of gene expression in multiple anthocyanin genes contributes to adaptive flower color evolution in *Mimulus aurantiacus*. Mol. Biol. Evol. 26:433-444.

Tuttle, E. M. 2003. Alternative reproductive strategies in the white-throated sparrow: behavioral and genetic evidence. Behav.Ecol. 14:425-432.

Twyford, A. D., M. A. Streisfeld, D. B. Lowry, and J. Friedman. 2015. Genomic studies on the nature of species: adaptation and speciation in *Mimulus*. Mol. Ecol. 24:2601-2609.

von Wettberg, E. J., M. L. Stanton, and J. B. Whittall. 2010. How anthocyanin mutants respond to stress: the need to distinguish between stress tolerance and maximal vigour. Evol. Ecol. Res. 12:457–476.

Waser, N. M., and M. V. Price. 1981. Pollinator choice and stabilizing selection for flower color in *Delphinium nelsonii*. Evolution 35:376-390.

Warren, J. and S. Mackenzie. 2001. Why are all colour combinations not equally represented as flower-colour polymorphisms? New Phytol. 151:237-241.

Wennersten, L. and A. Forsman. 2012. Population-level consequences of polymorphism, plasticity and randomized phenotype switching: a review of predictions. Biol. Rev. Camb. Philos. Soc. 87:756-767.

Wright, S. 1931. Evolution in Mendelian populations. Genetics *16:* 97-159.

Wu, C. A., D. B. Lowry, A. M. Cooley, t. K. M. Wrigh, Y. W. Lee, and J. H. Willis. 2007. *Mimulus* is an emerging model system for the integration of ecological and genomic studies. Heredity 100:220-230.

Wu, C. A., M. A. Streisfeld, L. I. Nutter, and K. A. Cross. 2013. The genetic basis of a rare flower color polymorphism in *Mimulus lewisii* provides insight into the repeatability of evolution. PloS one 8:e81173.

Yuan, Y. W., A. B. Rebocho, J. M. Sagawa, L. E. Stanley, and H. D. Bradshaw, Jr. 2016. Competition between anthocyanin and flavonol biosynthesis produces spatial pattern variation of floral pigments between Mimulus species. Proc. Natl. Acad. Sci. USA 113:2448-2453.

Yuan, Y. W., J. M. Sagawa, L. Frost, J. P. Vela, and H. D. Bradshaw. 2014. Transcriptional control of floral anthocyanin pigmentation in monkeyflowers (*Mimulus*). New Phytol. 204:1013-1027.

Table 1: Summary of REML mixed effect models on morphological traits in A) field experiment, B) growth-chamber experiment with two treatments (control and UV light) and C) growth chamber experiment with two treatments (control and drought). Plants were of known genotype for anthocyanin production. If the effect of genotype is significant, we present pairwise comparisons. Germination was not measured in the field experiment, and seed set was only measured in the field experiment. D) Magnitude of additive and dominant gene action in the field experiment for the two alleles at the single anthocyanin locus calculated on non-standardized data.

Source of variation	Germination	Flowering time	Leaf length	Stolons	Flowering branches	Seed set
A) Field Experiment						
Genotype		$F_{2,101}$ =18.82***	$F_{2,107}$ =1.06	$F_{2,92.8}$ =61.65***	$F_{2,92}$ =8.92**	$F_{2,11.54}$ =0.89
AA vs *Aa*		t_{101} =0.86		$t_{92.1}$ =3.09**	t_{92} =1.26	
AA vs *aa*		t_{101} =5.73***		$t_{93.2}$ =10.70***	t_{92} =2.74*	
Aa vs *aa*		t_{101} =4.80***		t_{93} =7.37***	t_{92} =4.08**	
Family [Genotype]		X^2_1=0.7	X^2_1=1.7	X^2_1=1.1	X^2_1=0	X^2_1=0
B) UV Experiment						
Genotype	$F_{2,185}$ =3.87*	$F_{2,9.04}$ =8.91**	$F_{2,9.09}$ =1.12	$F_{2,8.88}$ =4.72*	$F_{2,9.18}$ =0.42	
AA vs *Aa*	t_{185} =0.54	$t_{8.95}$ =0.72		$t_{8.85}$ =1.57		
AA vs *aa*	t_{187} =2.63*	$t_{9.13}$ =3.96*		$t_{8.91}$ =3.07*		
Aa vs *aa*	t_{185} =2.15*	$t_{9.03}$ =3.26*		$t_{8.87}$ =1.50		
Treatment	N/A	$F_{1,3.84}$ =9.82*	$F_{1,\ 3.79}$ =10.61*	$F_{1,\ 3.95}$ =0.93	$F_{1,\ 3.93}$ =0.71	
Genotype x Treat			$F_{2,\ 174}$ =7.07**	$F_{2,\ 170}$ =6.12**		
Family [Genotype]	X^2_1=0	X^2_1=15.2***	X^2_1=16.8***	X^2_1=21.9***	X^2_1=39.2 ***	
C) Drought Experiment						
Genotype	$F_{2,9}$ =0.48	$F_{2,9.18}$ =13.32**	$F_{2,9.14}$ =13.09**	$F_{2,182}$ =12.96***	$F_{2,9.22}$ =11.31*	
AA vs *Aa*		$t_{9.13}$ =0.39	$t_{9.02}$ =1.35	t_{181} =1.73	$t_{9.12}$ =2.64	
AA vs *aa*		$t_{9.29}$ =4.26*	$t_{9.26}$ =3.59*	t_{182} =5.01***	$t_{9.33}$ =6.13***	
Aa vs *aa*		$t_{9.13}$ =4.66**	$t_{9.12}$ =4.96**	t_{182} =3.31**	$t_{9.18}$ =3.52*	
Treatment		$F_{1,3.72}$ =16.56*	$F_{1,169}$ =17.50***	$F_{1,\ 3.98}$ =0.17	$F_{1,3.9}$ =18.91***	
Genotype x Treat				$F_{2,\ 182}$ =8.47**		

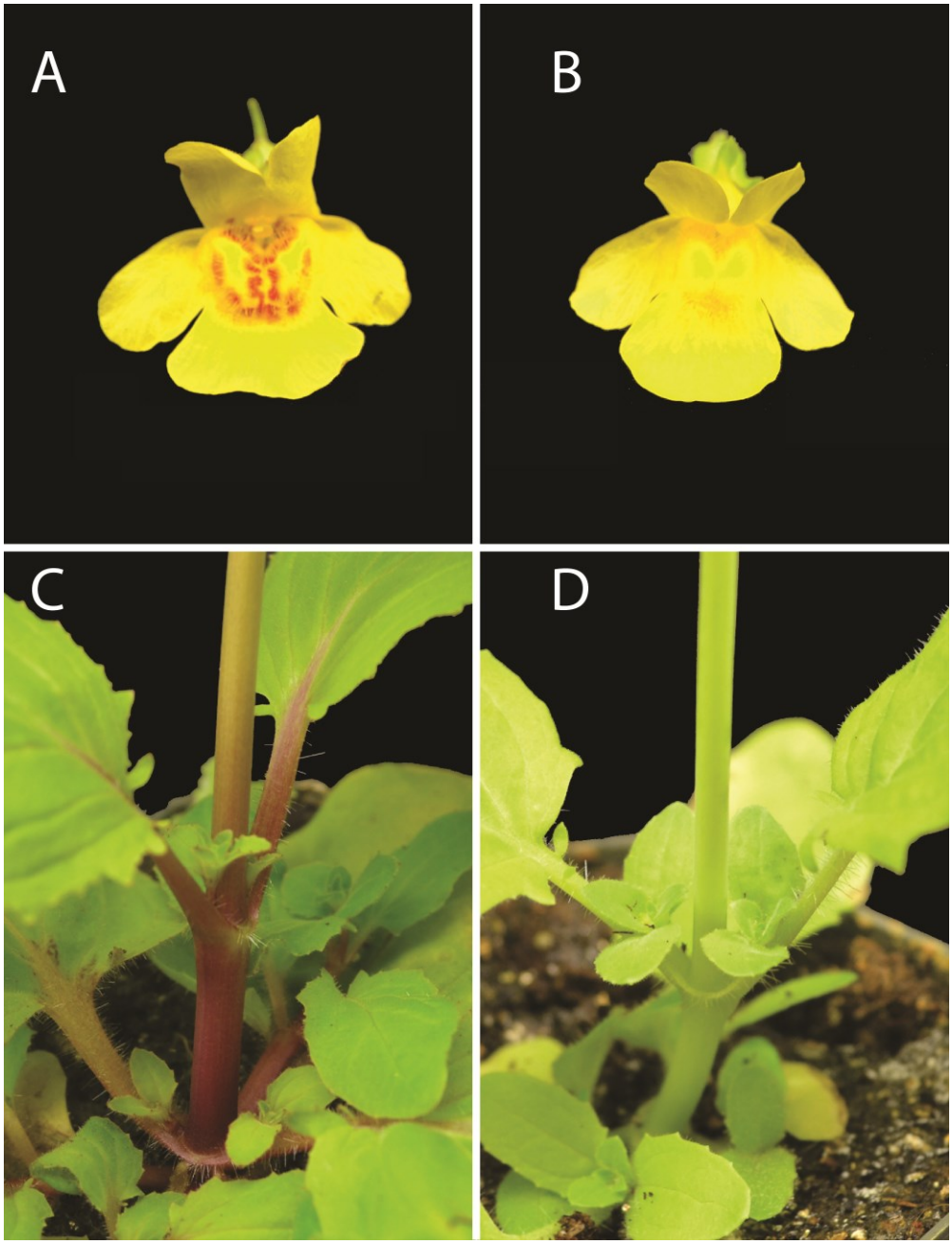
Family [Genotype]	X^2_1=1.6	X^2_1=10.2**	X^2_1=7.4*	X^2_1=1.8	X^2_1=15.16***	
D) Gene action						
A		4.71	0.04	4.71	1.21	23.38
D		3.29	0.42	3.29	1.98	10.70
d/a		0.70	11.5	0.70	1.65	0.46

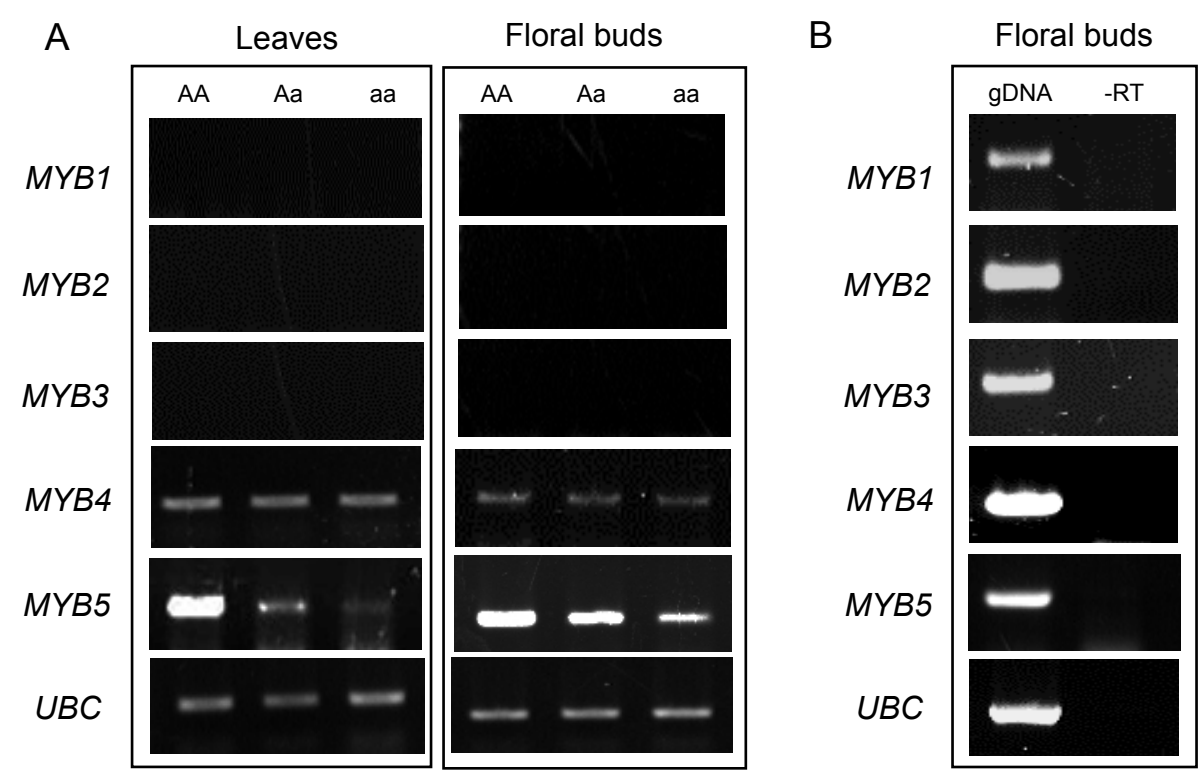
*** P < 0.0001; ** P < 0.001; * P<0.05

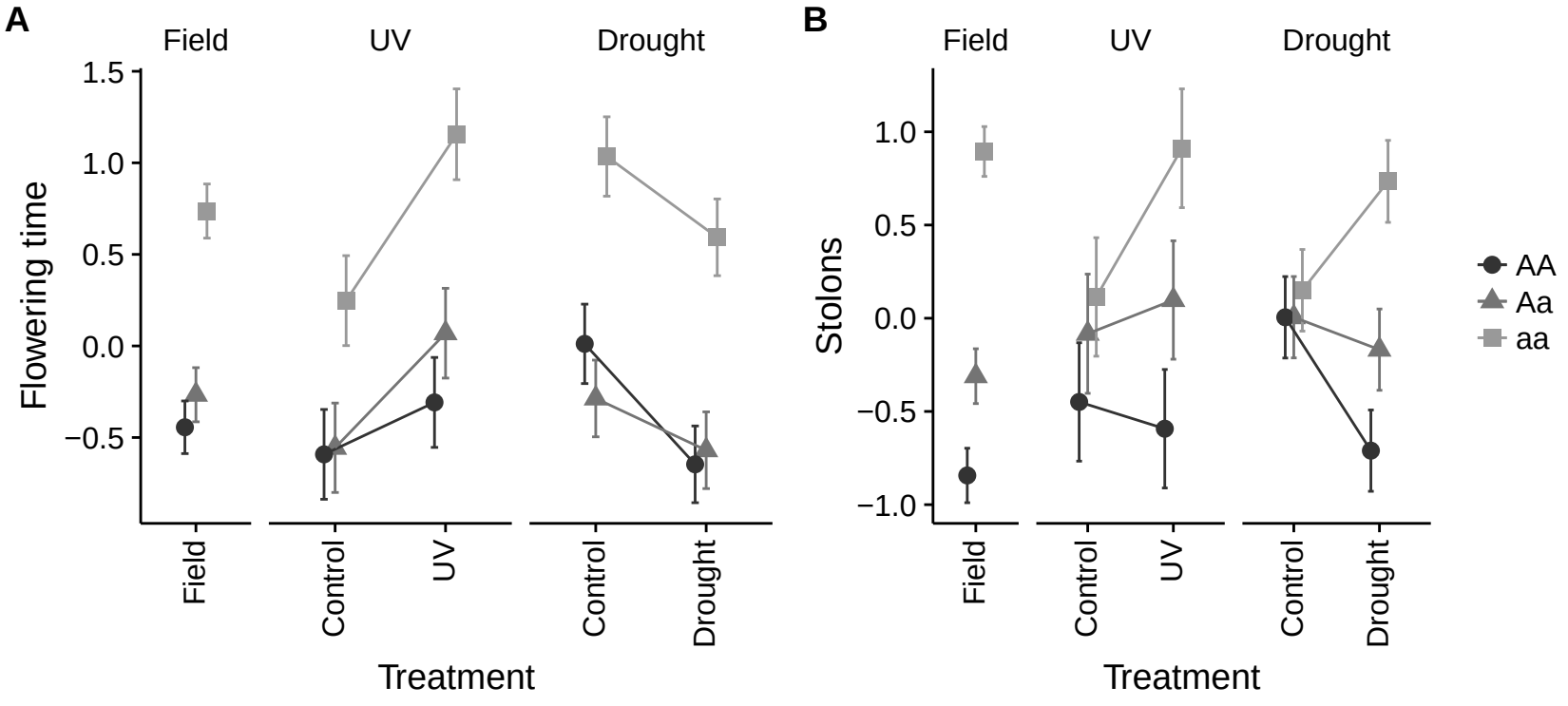
Figure 1: Photographs of representative *Mimulus guttatus:* A) pigmented (*AA*) and B) anthocyanin-deficient (*aa*) flowers, and c) pigmented and d) anthocyanin-deficient stems and leaves.

Figure 2: Gene expression analysis of *R2R3-MYB* genes in *M. guttatus*. A) Semi-quantitative RT-PCR analysis of *MYB1-5* genes in leaves, floral buds of plants with genotype *AA*, *Aa*, and *aa*. For RNA isolation leaves were pooled from four plants of each genotype. *UBC* was used as a normalization control for RNA concentration. B) Genomic DNA was used as a positive control and –RT (reaction without reverse transcriptase) was used as a negative control in RT-PCR experiments. Experiments were repeated with two independent biological samples of leaves and floral buds with similar results. PCR primers and cycle numbers are given in the appendix.

Figure 3: Influences of genotype and treatment on flowering time and stolon number. A) Flowering time and B) number of stolons in three experiments (Field, UV treatment, Drought treatment) with plants of known anthocyanin genotype. Standardized mean (± SE) data are shown. See Table 1 for statistical details.


A
B
C
D


A
Leaves
Floral buds
AA
Aa
aa
AA
Aa
aa
MYB1
MYB2
MYB3
MYB4
MYB5
UBC
B
Floral buds
gDNA
-RT
MYB1
MYB2
MYB3
MYB4
MYB5
UBC


A
Field
UV
Drought
Flowering time
1.5
1.0
0.5
0.0
−0.5
Field
Control
UV
Control
Drought
Treatment
B
Field
UV
Drought
Stolons
1.0
0.5
0.0
−0.5
−1.0
Field
Control
UV
Control
Drought
Treatment
AA
Aa
aa