How petals change their spots: cis-regulatory re-wiring in Clarkia (Onagraceae)

Talline R. Martins1,2, Peng Jiang1 and Mark D. Rausher1

1Department of Biology, Duke University, Durham, NC 27708, USA; 2Department of Agriculture, Nutrition and Veterinary Sciences, University of Nevada, Reno, NV 89557, USA

Summary

• A long-standing question in evolutionary developmental biology is how new traits evolve. Although most floral pigmentation studies have focused on how pigment intensity and composition diversify, few, if any, have explored how a pattern element can shift position. In the present study, we examine the genetic changes underlying shifts in the position of petal spots in Clarkia.
• Comparative transcriptome analyses were used to identify potential candidate genes responsible for spot formation. Co-segregation analyses in F2 individuals segregating for different spot positions, quantitative PCR, and pyrosequencing, were used to confirm the role of the candidate gene in determining spot position. Transient expression assays were used to identify the expression domain of different alleles.
• An R2R3Myb transcription factor (CgMyb1) activated spot formation, and different alleles of CgMyb1 were expressed in different domains, leading to spot formation in different petal locations. Reporter assays revealed that promoters from different alleles determine different locations of expression.
• The evolutionary shift in spot position is due to one or more cis-regulatory changes in the promoter of CgMyb1, indicating that shifts in pattern element position can be caused by changes in a single gene, and that cis-regulatory rewiring can be used to alter the relative position of an existing character.

Introduction

Elucidating how genetic changes alter developmental processes to generate phenotypic diversity has been one of the major objectives of evolutionary developmental biology (Hoekstra & Nachman, 2003; Wittkopp et al., 2003; Ferguson et al., 2011; Reed et al., 2011). Color patterns in both animals and plants frequently have been employed as model systems for addressing this issue (Hoekstra & Nachman, 2003; Gompel et al., 2005; Mundy, 2005; Schwin et al., 2006; Reed et al., 2011; Martin et al., 2012; Sobel & Streisfeld, 2013). There are two primary ways in which color patterns can differ among species: (1) a pattern element may be present in one species but absent in a second (Gompel et al., 2005; Kodandaramaiah, 2009; Wallbank et al., 2016); or (2) the relative positions of different pattern elements may differ (Nijhout, 2001; Saenko et al., 2011; Ellis et al., 2013). Prior investigations of pattern evolution in organisms as diverse as Drosophila, Heliconius butterflies, mice, birds and flowering plants, have focused almost entirely on the genetic and developmental underpinnings of mode (1) and have demonstrated that cis-regulatory changes often contribute to gain or loss of pattern elements. By contrast, much less is known about evolutionary shifts in the positions of pattern elements, particularly in plants. Although a number of studies have shown that changes in the spatial expression domain of gene products that control or initiate pattern element formation are associated with changes in the position of those elements (Bartlett & Specht, 2011; Saenko et al., 2011; Hileman et al., 2013), we are unaware of any studies that have elucidated the mechanisms responsible for change in expression domain. The answers thus remain unknown for basic questions such as whether position shifts tend to involve many or few genes, whether they are caused by changes in gene-coding regions or regulatory regions, or whether they involve the same kinds of genetic change as gain or loss of elements. In the present study, we address these questions by examining the genetic changes involved in the evolutionary alteration of petal spot position in the genus Clarkia (Onagraceae).

Petal spot position in Clarkia is an ideal system for distinguishing between these two mechanisms of trait evolution. Petal spots have evolved repeatedly in the flowering plants. Experimental studies and observations indicate that variation in petal spot pattern can alter pollinator visitation rates and male reproductive success, including in Clarkia species (Jones, 1996; Eckhart et al., 2006; Ellis & Johnson, 2010). Previous research has shown that species in the section Rhodanthos have diverged in the position of the floral spot (Gottlieb & Ford, 1988) (Fig. 1). In most species, the spot is located at the base of the petal, representing the ancestral condition (Martins, 2011), but one species,
activators regulate them (Fig. 2b). We define pre-pattern activators as the collection of upstream regulatory genes – positive and negative regulators – that determine where the developmental initiators are expressed. Alternatively, alteration of the expression domain of pre-pattern activators (Fig. 2c), or acquisition, by previously unused upstream regulatory genes, of promoter binding sites for the developmental initiators (Fig. 2d), are two ways that genetic changes in the pre-pattern activators could alter spot position. Unfortunately, little is known about the identity of these pre-pattern activators for the anthocyanin pathway in plants. Petal spots in *Clarkia* thus provide an opportunity to distinguish cleanly between changes in a developmental initiator and in a pre-pattern activator as explanations for the evolutionary shift in spot position.

In the present study, we show that this evolutionary change in the genus *Clarkia* (Onagraceae) occurred by a cis-regulatory modification of an *R2R3Myb* anthocyanin pathway transcriptional regulator, a developmental initiator. This change alters the gene’s expression domain and directly shifts the position where spot-forming anthocyanins accumulate.

**Materials and Methods**

**Transcriptomics and gene expression levels**

RNASeq was performed for *Clarkia gracilis* (Piper) A. Nelson & J. F. Macbride for four types of tissues: (1) basal petal spots; (2) petal background from petals with basal spots; (3) central petal spots; and (4) petal background from petals with central spots. Tissues (1) and (2) were from *C. g. albicaulis*, and tissues (3) and (4) were from *C. g. sonomensis*. RNA was extracted from each tissue type using the RNaseasy Plant Mini kit (Qiagen). Illumina libraries with c. 300-bp inserts were created and 100 paired end reads were obtained (estimate coverage of 160X per library) on an Illumina HiSeq 2000 platform at the Duke Center for Genomic and Computational Biology. Quality control and trimming of Illumina reads were performed using the Trimmomatic software (Bolger et al., 2014) before transcriptome assembly. Transcriptomes were assembled using Trinity software (Grabherr et al., 2011) for two samples: central-spotted plant (spotted + unspotted region) and basal-spotted plant (spotted + unspotted region). Gene expression levels were estimated as FPKM values separately for each tissue (basal and central, spotted and unspotted) using the RSEM software built into the Trinity package (Li & Dewey, 2011) against respective transcriptomes. All transcriptomic analyses were performed in the XSEDE virtual computing system (Towns et al., 2014).

For gene expression analyses, petals were cut into three separate sections, with one section corresponding to the position of the basal spot (basal c. 1/3 of petal), one corresponding to the position of the central spot (c. middle 1/3 of petal), and one corresponding to the unspotted portion (c. distal 1/3 of petal). These three sections were obtained for each of the three phenotypes: petals with basal spots, petals with central spots, and petals with both basal and central spots. RNA was extracted from petal sections using the RNaseasy Plant Mini kit (Qiagen), and cDNA was...
synthesized using the M-MLV reverse transcriptase (Invitrogen). Quantitative real-time PCR (qPCR) was performed on an Eppendorf Mastercycler RealPlex, using the Dynamo SYBR green qPCR mix (Finnzymes, Woburn, MA, USA). Three biological replicates for each phenotype and two technical replicates for each sample were performed. Levels of *Actin* gene expression were determined to be constant in all four Illumina libraries, unlike *Ef1-a*, which was expressed at different levels in basal vs central samples. Therefore, *Actin* was chosen as a standard to which expression levels were relativized. The following cycling conditions were used: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s. A dissociation curve was run at the end of the cycle to verify that a single product was amplified. Primer efficiencies and relative levels of expression were estimated as previously described (Peirson *et al.*, 2003; Streisfeld & Rausher, 2009). Primers used for *Actin* amplification were forward 5'CACGAAAACCACCTACAACTCG3' and reverse 5'GAGATCCACATCGTTGGAAAG3'. Primers used for *CgMyb1* were forward 5'AGCAGGTTTGAATAGGTGCA3' and reverse 5'TCCTCCTGCTATGAGTGACCA3'.

*CgMyb1* cloning, SNP identification, allele-specific expression and genotyping

The complete coding sequence for *CgMyb1* was obtained from transcriptome samples and confirmed by Sanger sequencing. Sequence comparison revealed single nucleotide polymorphisms (SNPs) that were used to distinguish among *C. g. albicaulis* and *C. g. sonomensis* alleles for estimating relative expression levels of the two alleles. A SNP at position 405 was evaluated using the primers forward 5'CGCCATAATAAGGCCTATTGCA3' and reverse (biotinylated) 5'TCATCCTGCTATCAACTCG3'. The relative expression levels in double-spotted individuals were quantified using pyrosequencing. Pyrosequencing was performed using three biological and four technical replicates in a PSQ HS 96A system. The sequencing primer used was forward 5'GCCCCCTATTGGCGCTTTG3' and reverse (biotinylated) 5'TCATCCTGCTATGAGTGACCA3'. The relative expression levels in double-spotted individuals were quantified using pyrosequencing. Pyrosequencing was performed using three biological and four technical replicates in a PSQ HS 96A system. The sequencing primer used was forward 5'GCCCATAATAAGGCCTATTGCA3' and reverse (biotinylated) 5'TCATCCTGCTATCAACTCG3'. The relative expression levels in double-spotted individuals were quantified using pyrosequencing. Pyrosequencing was performed using three biological and four technical replicates in a PSQ HS 96A system. The sequencing primer used was forward 5'GCCCATAATAAGGCCTATTGCA3' and reverse (biotinylated) 5'TCATCCTGCTATCAACTCG3'. The relative expression levels in double-spotted individuals were quantified using pyrosequencing. Pyrosequencing was performed using three biological and four technical replicates in a PSQ HS 96A system. The sequencing primer used was forward 5'GCCCATAATAAGGCCTATTGCA3' and reverse (biotinylated) 5'TCATCCTGCTATGAGTGACCA3'.

**Fig. 2** Possible mechanisms for shift in petal spot position. (a) Ancestral condition. Two sets of pre-pattern activators PPA-X and PPA-Y are expressed in different petal spatial domains. PPA-X activates the developmental initiator, *Di* (designated allele *Dp*, for basal spot) by binding to *Dp* cis-regulatory element(s) portrayed schematically as orange oval. *Dp* activates the enzyme-coding genes of the anthocyanin biosynthetic pathway (ABP), which in turn produces a basal spot. (b) One or more cis-regulatory mutations (green oval) causes *Di* (allele now designated *Dp* to reflect regulatory changes) to be activated by a different set of pre-pattern activators (PPA-Y) that are expressed in the central part of the petal. *Dp* still activates the ABP and causes spot formation in the central part of the petal. (c) Mutations cause PPA-X expression domain to shift to the central part of the petal. PPA-X activates *Di* (still designated *Dp* because there has been no genetic change to this gene), which activates the ABP, producing a central spot. (d) One or more mutations to pre-pattern PPA-Y cause it to bind to a different site within the promoter region of *Dp* (purple oval), activating that gene and in turn the ABP, producing a central spot. There may have been a double-spotted evolutionary intermediate if pre-pattern PPA-X also activated *Myb1*. Subsequent elimination of this activation (‘X’ in figure) could be due to mutations either in PPA-X or the cis-regulatory region of *Di*. Green and orange areas around PPA-X and PPA-Y represent possible expression locations for these genes.


© 2016 The Authors

New Phytologist © 2016 New Phytologist Trust

www.newphytologist.com
In order to generate promoter-driven luciferase (LUC) reporter constructs, we introduced a 15-bp extension homologous to vector sequences into the promoter DNA fragment (c. 1000 bp) of the \( CgMyb1 \) and \( CgMyb1 \) genes by PCR amplification. The promoter fragments were then cloned into a reconstituted binary plasmid \( pRZ500-35S-LUC \) (Zhang et al., 2011) using standard cloning methods. All of the plant promoter-reporter vectors constructed were confirmed by sequencing, and then transformed into \( Agrobacterium\) tumefaciens strain GV3101 by the freeze–thaw method. \( Agrobacterium\)-mediated transient expression of promoter::LUC constructs were conducted on healthy and fully expanded petals of 12-wk-old \( C. gracilis \) plants. The transformed \( Agrobacteria\) cells, Ag/pGL-basic were grown on Luria-Bertani broth containing 30 \( \mu \)g ml\(^{-1}\) rifampicin and 50 \( \mu \)g ml\(^{-1}\) kanamycin at 28\(^\circ\)C for 42 h in an incubator with shaking at 200 rpm. The cells were harvested by spinning at 2000 \( g \) for 20 min at 6\(^\circ\)C. The \( Agrobacteria\) pellet was resuspended in autoclaved distilled water to cultures with absorbance values of 0.3, 0.6 and 1.0 at a 600 nm wavelength using Biophotometer Plus (Eppendorf, Germany).

An equal amount of each culture was vacuum-infiltrated into both sides of \( C. albicaulis \) (basal-spotted) or \( C. sonomensis \) (central-spotted) petals for each group of assays. Vacuum infiltration was initiated by placing the petals and culture into a 50 ml tube and subjecting the tube to a vacuum. The vacuum was applied to reach an absolute pressure of 0.23 atm for 3 min and then released to let the \( A. tumefaciens\) culture infiltrate into the petals. The infiltrated petals were allowed to air-dry in a biological safety cabinet for c. 15 min, and were subsequently allowed to rest in an artificial growth room in darkness for 12 h. Afterwards, they were incubated under 16 h : 8 h, light : dark for a total of 36 h at 25\(^\circ\)C. Petals were kept in a Petri-dish with a wet paper towel inside to preserve moisture. Each vacuum infiltration treatment was done in triplicate. The LUC activity was visualized with a Lumazon imaging system (MAG Biosystems, Santa Fe, NM, USA).

Results

The spot-formation gene is an R2R3 MYB transcription factor

Because interspecific crosses in \( Clarkia\) are often problematic, it is not possible to identify genes associated with differences in spot position using mapping populations derived from between-species crosses. However, such identification is possible using the tetraploid \( C. gracilis\). This species has four distinct but interfertile subspecies among which the position of the petal spot varies (Fig. 1a). This species is believed to be an allotetraploid that was formed by a cross between the central-spotted \( C. amoenus\) and an extinct relative of the basal-spotted \( C. lassenensis\) and \( C. arcuata\) (Fig. 1b) (Abdel-Hameed & Snow, 1972). Previous work established that the different spot positions in different subspecies are determined by different alleles at the \( P \) locus (Gottlieb & Ford, 1988). Specifically, two alleles are found at this locus: \( P^a \) and \( P^b\). These alleles determine whether basal or central spots form, respectively, in homozygotes. Plants with a \( P^aP^a \) genotype develop both central and basal spots (a double-spotted flower). This result suggests that the novel central spot position evolved via a substitution at a single locus.

In order to identify the gene underlying the \( P \) locus and the type of substitution(s) involved, we sequenced and assembled the transcriptomes of spotted and unspotted sections of petals from two different subspecies of \( C. gracilis\) – the basal-spotted ssp. \( albicaulis\) and the central-spotted ssp. \( sonomensis\) – and quantified transcript expression levels. Genes whose expression levels were found to be at least ten times higher in spotted sections compared with unspotted sections in both subspecies were identified as potential candidates for the \( P \) locus.

The top 20 most differentially expressed genes in both subspecies contained several genes involved in anthocyanin
biosynthesis (Supporting Information Table S1). One of these was $CgDfr2$, which codes for the anthocyanin-pathway enzyme dihydro-flavonol reductase. This gene has been shown to be expressed only in petal spots, whereas the paralog $CgDfr1$ is expressed in the petal background (Martins et al., 2013). A second gene, which we designate $CgAns$, BLASTs most closely to anthocyanidin synthases from a variety of angiosperm species. Finally, also among the top 20 most differentially expressed genes was an $R2R3Myb$ transcription factor, which we designate $CgMyb1$, and which blasted most closely to the Petunia AN2 locus, a transcriptional activator of the enzyme-coding genes in the anthocyanin biosynthetic pathway (Quattrocchio et al., 1993). Orthologs of this gene have been shown to activate anthocyanin-pathway enzyme-coding genes in other species as well (Quattrocchio et al., 1993; Schwinn et al., 2006; Streisfeld et al., 2013).

Expression levels from the transcriptome samples indicate that $CgMyb1$ is expressed over 100× higher in spotted sections of petals than in unspotted sections, regardless of spot location (Table S1). We confirmed this difference in expression level using qPCR (Fig. 3a). $CgMyb1$ is expressed at equal levels in the two spots. The spot-specific pattern of expression is similar to that of $CgDfr2$ (Martins et al., 2013), suggesting that $CgMyb1$ may activate $CgDfr2$ and $CgAns$, as well as other anthocyanin enzyme-coding genes, to produce spots.

Two other lines of evidence support this conclusion. First, in $F_2$ individuals of a cross between $C. g. albicaulis$ (basal spots) and $C. g. sonomensis$ (central spots), spot position co-segregates perfectly in a 1 : 2 : 1 ratio with parental alleles of $CgMyb1$ (distinguishable by several SNPs, Fig. S1), with genetic heterozygotes producing both basal and central spots (Fig. 4a). Second, a subspecies lacking spots altogether, $C. g. gracilis$, has a $CgMyb1$ allele with a frame-shift deletion in the R2R3 DNA binding domain that presumably renders it nonfunctional (Fig. S1). This inference was confirmed by examining the $F_2$ progeny of a cross between this unspotted subspecies and $C. g. albicaulis$ (basal spots): there was perfect co-segregation between phenotype and $CgMyb1$ genotype (Fig. 4b), which is expected if $CgMyb1$ activates anthocyanin enzyme-coding genes in spots.

Different alleles of $CgMyb1$ have different expression domains

Using pyrosequencing, we quantified allele-specific expression levels of $CgMyb1$ in the basal and central spots of heterozygotes. Each allele was expressed primarily at only one spot position: allele $CgMyb1^b$, from the parent with basal spots, constituted >90% of the transcripts in basal spots; by contrast, $CgMyb1^c$, from the parent with central spots, constituted >90% of the transcripts in central spots (Fig. 3b).

This differential expression implicates a change in the cis-regulatory regions of $CgMyb1$ as the cause of the shift from basal to central spot position (Fig. 2b), rather than a genetic change in pre-pattern genes (Fig. 2c,d). We confirmed this inference by functional analysis of promoters of the two alleles. When infiltrated into developing petals with either basal or central spots, reporter gene activity for the $CgMyb1^b$ promoter was localized to the basal region of the petal (Fig. 5a,b), whereas activity for the $CgMyb1^c$ promoter was localized to the central region of the petal (Fig. 5c,d). This pattern held regardless of whether the spot itself was expressed, indicating that promoter expression was driven by pre-pattern activators rather than by genes that are part of the anthocyanin biosynthetic pathway, including its developmental initiators.

The central spot allele of $CgMyb1$ appears to have evolved once in section Rhodanthos

Ancestral character reconstruction analyses suggest that the basal spot is the ancestral condition in section Rhodanthos of the genus Clarkia (Fig. 1). Central spots are present in only two species in section Rhodanthos: $C. amoena$ and $C. gracilis$ ssp. sonomensis. To
ortholog of the CgMyb1c allele. If these substitution(s) did not themselves eliminate recognition of the original set of transcription factors (PPA-X), this was subsequently achieved by additional regulatory mutations. This model explains the evolutionary shift in spot position to have been achieved by an alteration of the domain of expression of CgMyb1 through cis-regulatory rewiring.

One potential problem with this model is that the evolution of spot position in the diploid species is inferred from analysis of the tetraploid C. gracilis. In particular, the difference in spot patterns between the central-spotted diploid species C. amoena and the basal-spotted diploid species that was the other progenitor of C. gracilis, is envisioned to be caused by different alleles at a diploid locus. When the two progenitor species crossed to form the tetraploid C. gracilis, it is possible that the chromosomes containing CgMyb1 paired as tetravalents at meiosis. When the tetrasomy subsequently was resolved into disomic inheritance, CgMyb1 may have segregated for the two parental alleles. However, allotetraploids usually exhibit disomic inheritance at their origin (Meirmans & Van Tienderen, 2013). If this were true for C. gracilis, then the parental copies of CgMyb1 initially would have resided at different loci on different chromosomes. How, then, could the two copies have become allelic at a single locus? We can envision two possibilities: (1) there was an independent mutation at one of the loci that created a new ‘central-spotted’ allele, accompanied by deletion of the second locus; and (2) there was a gene conversion event, whereby one copy of the allele at one locus was converted to the allele at the other locus, followed by deletion of the second locus. Both scenarios would have resulted in individuals heterozygous for the ‘basal’ and ‘central’ alleles at a single locus. This variation could then have been alternatively sorted into the different subspecies, followed by fixation of the loss-of-function mutation in the ‘basal’ allele in C. gracilis to eliminate spot formation.

These two alternative scenarios can be differentiated by examining relationships between alleles from different species. Alternative (1) predicts that the ‘basal’ and ‘central’ alleles from C. gracilis should be most closely related to each other, whereas alternative (2) predicts that each should be more closely related to the alleles from species with spots in similar positions. The actual gene tree supports alternative (2), with the ‘central’ allele from C. gracilis being most closely related to the allele from C. amoena, and the ‘basal’ allele from C. gracilis being most closely related to the alleles from the basal-spotted species (Fig. 6). The strong bootstrap support for the C. gracilis-C. amoena clade indicates that the two alleles from C. gracilis are not most closely related to each other, ruling out alternative (1). It thus seems likely that if C. gracilis exhibited disomic inheritance immediately upon being created by hybridization, diploidization of the two CgMyb1 alleles involved a gene conversion event.

In plants, the production of anthocyanin pigments is activated by a complex of three transcription factors that includes an R2R3Myb gene (Ramsay & Glover, 2005). Evolutionary changes in floral pigment patterns caused by elimination of anthocyanin pigmentation from flowers, or from parts of flowers, typically

determine whether the CgMyb1c allele evolved in the tetraploid C. gracilis de novo, or whether it was inherited through one of its purported diploid parents, C. amoena, we sequenced near full-length Myb1 from other diploid members of the Rhodanthos clade and estimated the evolutionary history of the CgMyb1c allele. We found that the CgMyb1c allele is more closely related to the C. amoena Myb1 allele than to the CgMyb1d present in other C. gracilis ssp (Fig. 6), suggesting that the central spot present in C. gracilis ssp. sonomensis is a result of inheritance of the Myb1 allele from C. amoena during polyploid formation.

Discussion

Our results suggest that CgMyb1 is the developmental initiator (DI) in our model (Fig. 1) and the following developmental and evolutionary model for a shift in spot position: ancestrally in the Rhodanthos clade, basal spots were generated by expression of an ortholog of the CgMyb1c allele (Fig. 1a). Expression of this allele, which was restricted to the area of the basal spot, presumably was activated by one or more unknown transcription factors whose domain of expression was limited to that area (PPA-X in Fig. 2a).

In the lineage leading to the central-spotted Clarkia amoena, one or more substitutions occurred in the gene’s regulatory region(s) that caused CgMyb1 to be activated by a new set of transcription factors (PPA-Y in Fig. 2b) whose domain of expression corresponded to the area of the central spot, converting it to an
involve downregulation or inactivation of the R2R3Myb even though mutations in at least eight other genes can produce the same phenotype (Streisfeld et al., 2013). These changes do not, however, involve regulatory rewiring. By contrast, our results indicate that cis-regulatory rewiring of CgMyb1 is a likely cause of a shift in the position of petal spots in Clarkia, without any change to regulatory genes that constitute the ancestral and derived pre-patterning activators. A remaining challenge is to identify the exact nature of that rewiring, including the original and new pre-patterning activators that regulate this gene. The current study does not provide specific candidates for the pre-pattern activators; however, it is likely that genes with roles in petal development and patterning are involved. Some potential binding sites were identified in the regulatory region of both CgMyb1 alleles, and merit further investigation (Fig. S2; Table S2). It is also important to note that, although we use the term pre-pattern activators, it is possible that the simultaneous action of positive as well as negative regulators lead to CgMyb1’s pattern of expression, as seen with wing-spots in Drosophila (Gompel et al., 2005). Investigations on the nature of pre-patterns may shed a light on which types of pigmentation patterns are more and less likely to evolve.

Acknowledgements

We thank Fred Nijhout for advice and comments on the manuscript. Support was provided by NSF grants DEB-0841521 and DEB-1542387 to M.D.R. and DBI-1103693 to T.R.M. Rodolfo Zentilla Gomez provided the binary plasmid. This work used the Extreme Science and Engineering Discovery
Author contributions
T.R.M performed the crosses, genotyping, transcriptome assemblies and gene expression assays; P.J. performed the luciferase assays; T.R.M. and M.D.R. conceived the project, designed the experiments and analyzed the data; all three authors wrote the paper.

References

**Fig. S1** Alignment of *CgMyb1*<sup>1</sup>, *CgMyb1*<sup>2</sup> and *CgMyb1*<sup>3</sup> showing frame-shift deletion in exon 1 of *CgMyb1*<sup>1</sup>.

**Fig. S2** Alignment of *CgMyb1*<sup>1</sup> and *CgMyb1*<sup>2</sup> upstream regulatory regions used for luciferase assays.

**Table S1** Top 20 most differentially expressed genes in bas al-spotted and central-spotted *Clarkia gracilis*

**Table S2** Candidate *cis*-acting regulators of *CgMyb1* alleles

Please note: Wiley Blackwell are not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.