

An R2R3-MYB transcription factor regulates carotenoid pigmentation in *Mimulus lewisii* flowers

Janelle M. Sagawa^{1*}, Lauren E. Stanley^{1*}, Amy M. LaFountain², Harry A. Frank², Chang Liu¹ and Yao-Wu Yuan^{1,3}

¹Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, CT 06269, USA; ²Department of Chemistry, University of Connecticut, Storrs, CT 06269, USA;

³Institute for Systems Genomics, University of Connecticut, Storrs, CT 06269, USA

Summary

Author for correspondence:

Yao-Wu Yuan

Tel: +1 860 486 3469

Email: yuan.colreeze@gmail.com

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- Carotenoids are yellow, orange, and red pigments that contribute to the beautiful colors and nutritive value of many flowers and fruits. The structural genes in the highly conserved carotenoid biosynthetic pathway have been well characterized in multiple plant systems, but little is known about the transcription factors that control the expression of these structural genes.
- By analyzing a chemically induced mutant of *Mimulus lewisii* through bulk segregant analysis and transgenic experiments, we have identified an R2R3-MYB, Reduced Carotenoid Pigmentation 1 (RCP1), as the first transcription factor that positively regulates carotenoid biosynthesis during flower development.
- Loss-of-function mutations in *RCP1* lead to down-regulation of all carotenoid biosynthetic genes and reduced carotenoid content in *M. lewisii* flowers, a phenotype recapitulated by RNA interference in the wild-type background. Overexpression of this gene in the *rcp1* mutant background restores carotenoid production and, unexpectedly, results in simultaneous decrease of anthocyanin production in some transgenic lines by down-regulating the expression of an activator of anthocyanin biosynthesis.
- Identification of transcriptional regulators of carotenoid biosynthesis provides the 'toolbox' genes for understanding the molecular basis of flower color diversification in nature and for potential enhancement of carotenoid production in crop plants via genetic engineering.

Introduction

Carotenoids are the most widely distributed pigments in nature, found in many bacteria, fungi, animals, algae, and virtually all plants. In all photosynthetic organisms, carotenoids are essential components of the photosynthesis system, where they carry out indispensable functions in protecting the photosynthetic apparatus from photooxidative damage and act as accessory pigments in light harvesting (Demmig-Adams *et al.*, 1996; Pogson *et al.*, 1998; Niyogi, 1999; Baroli & Niyogi, 2000; Dall'Osto *et al.*, 2007). In flowering plants, carotenoids are also synthesized as secondary metabolites and accumulate to high concentrations in chromoplasts, providing the distinct yellow, orange, and red colors to many flowers (e.g. daffodils, daylilies, sunflowers) and fruits (e.g. oranges, tomatoes, mangoes), and thus serving an important function in the ecology and evolution of plants by attracting pollinators and seed dispersers (Glover, 2014).

Unlike carotenoids in photosynthetic tissues, the carotenoids accumulated in chromoplasts are dispensable for plant growth and development. As a consequence, the composition, intensity, and spatial patterning of carotenoid pigments in flowers and

fruits can differ substantially between closely related species or even between different varieties of the same species (Moebs *et al.*, 2001; Bradshaw & Schemske, 2003; Nielsen *et al.*, 2003; Giovannoni, 2007; Ha *et al.*, 2007; Chiou *et al.*, 2010; Yamagishi *et al.*, 2010; Yamamizo *et al.*, 2010). Numerous studies have shown that the diversity of carotenoid pigmentation in flowers and fruits is largely determined by differential expression of carotenoid biosynthetic genes at the transcriptional level; and often this differential expression involves coordinated up- or down-regulation of multiple structural genes or the entire carotenoid biosynthetic pathway (CBP; Moebs *et al.*, 2001; Ha *et al.*, 2007; Chiou *et al.*, 2010; Yamagishi *et al.*, 2010; Yamamizo *et al.*, 2010), indicating the critical role of transcription factors (TFs) in the generation of color variation. However, while the structural genes on the highly conserved CBP (Fig. 1) have been well characterized in multiple plant systems (Bramley *et al.*, 1992; Pecker *et al.*, 1992; Cunningham *et al.*, 1996; Marin *et al.*, 1996; Sun *et al.*, 1996; Bugos & Yamamoto, 1996; Bartley *et al.*, 1999; Isaacson *et al.*, 2002; Tian *et al.*, 2004; Kim & DellaPenna, 2006; North *et al.*, 2007; Chen *et al.*, 2010; reviewed in Ruiz-Sola & Rodríguez-Concepción, 2012), little is known about the TFs that regulate the expression of these structural genes during flower or fruit development.

*These authors contributed equally to this work.

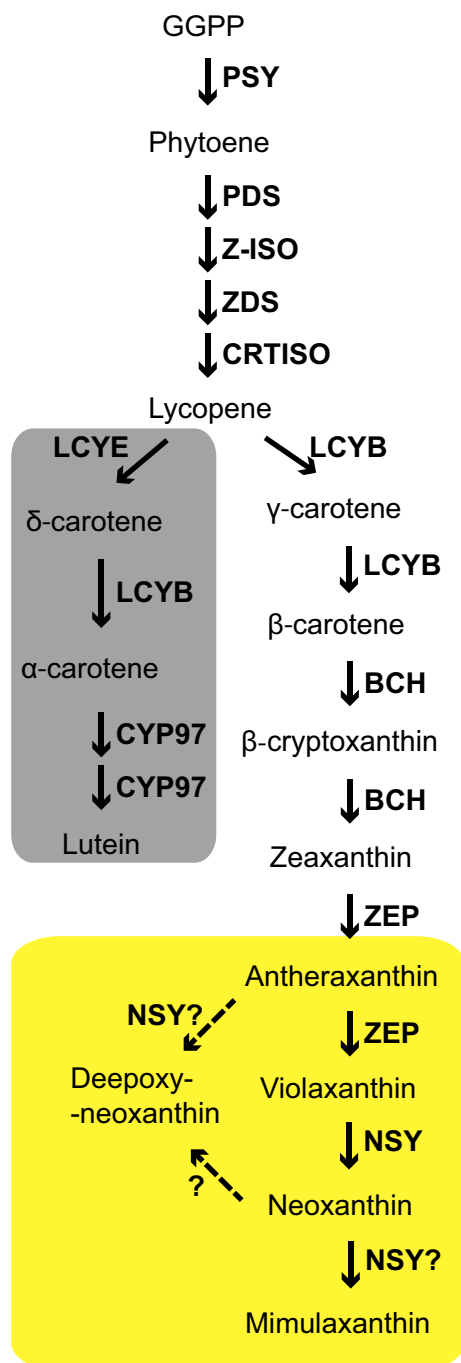


Fig. 1 A schematic of plant carotenoid biosynthetic pathway. The enzymes are shown to the side of the arrows. The α -carotene branch (shaded in gray) was not considered in this study because all *Mimulus lewisii* floral carotenoids (highlighted by the yellow background) are on the β -carotene branch (LaFountain *et al.*, 2015). GGPP, geranylgeranyl diphosphate; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, 15-cis- ζ -carotene isomerase; ZDS, ζ -carotene desaturase; CRTISO, carotenoid isomerase; LCYB, lycopene β -cyclase; LCYE, lycopene ϵ -cyclase; CYP97, cytochrome P450 family 97; BCH, β -carotenoid hydroxylase; ZEP, zeaxanthin epoxidase; NSY, neoxanthin synthase. Question marks indicate uncertainties in the pathway.

The transcriptional control of carotenoid accumulation in chromoplasts has been most extensively studied in tomatoes because of the dramatic color changes during fruit ripening (as

a result of lycopene accumulation) and the availability of abundant fruit color mutants. Several TFs have been implicated in the regulation of carotenoid accumulation during tomato fruit ripening, including the MADS-box proteins RIN, TAGL1, and TDR4 (Vrebalov *et al.*, 2002, 2009; Itkin *et al.*, 2009; Bemer *et al.*, 2012), the SQUAMOSA promoter binding protein CNR (Manning *et al.*, 2006), the HD-Zip homeobox protein Le-HB1 (Lin *et al.*, 2008), the AP2/ERF family protein SlAP2a and SIERF6 (Chung *et al.*, 2010; Lee *et al.*, 2012), and the NAC domain proteins NOR and SINAC4 (Martel *et al.*, 2011; Zhu *et al.*, 2014). However, all these TFs have broad ripening effects (e.g. ethylene synthesis, fruit softening, aroma and flavor production), and they are therefore unlikely to be specific regulators of carotenoid biosynthesis. Some of these TFs have been shown to directly interact with promoters of ethylene biosynthesis genes (e.g. Le-HB1, TAG1, RIN; Lin *et al.*, 2008; Itkin *et al.*, 2009; Fujisawa *et al.*, 2011), and ethylene is known to induce carotenoid biosynthesis during tomato fruit ripening (Su *et al.*, 2015), suggesting that these TFs may regulate carotenoid production indirectly through ethylene signaling. Among these TFs, only the MADS-box protein RIN has been implicated in direct regulation of one of the carotenoid biosynthetic genes, *phytoene synthase* (*PSY*), by a chromatin immunoprecipitation (ChIP) assay (Martel *et al.*, 2011). However, another study employing a similar strategy did not recover significant enrichment of the *PSY* promoter fragments after ChIP with RIN antibodies (Fujisawa *et al.*, 2011). Furthermore, mutations in these fruit ripening TFs do not cause any notable changes in tomato flower color, which is also carotenoid pigmented (Lin *et al.*, 2008; Vrebalov *et al.*, 2009; Chung *et al.*, 2010).

The regulation of carotenoid pigmentation in flowers is even less well understood – not a single TF regulating carotenoid pigmentation during flower development has been reported to date. In this study we employ the emerging genetic model system, *Mimulus lewisii* (Yuan *et al.*, 2013a,b, 2014), to address this problem. The ventral (lower) petal of *M. lewisii* flowers has two yellow ridges that are pigmented by carotenoids (marked by the red arrows in Fig. 2a), providing contrast against the anthocyanin-pigmented petal lobes (Fig. 2a). These yellow ridges have been demonstrated to act as nectar guides for bumblebee pollinators (Owen & Bradshaw, 2011). Through bulk segregant analysis of a chemically induced mutant and transgenic experiments, we have identified an R2R3-MYB TF, Reduced Carotenoid Pigmentation 1 (RCP1), as a master regulator of carotenoid biosynthesis during *M. lewisii* flower development.

Materials and Methods

Plant materials

The *Mimulus lewisii* Pursh inbred line LF10 (wild-type) and the mapping line SL9 were described in Yuan *et al.* (2013a,b). Ethyl methanesulfonate (EMS) mutants were generated using LF10, following Owen & Bradshaw (2011).

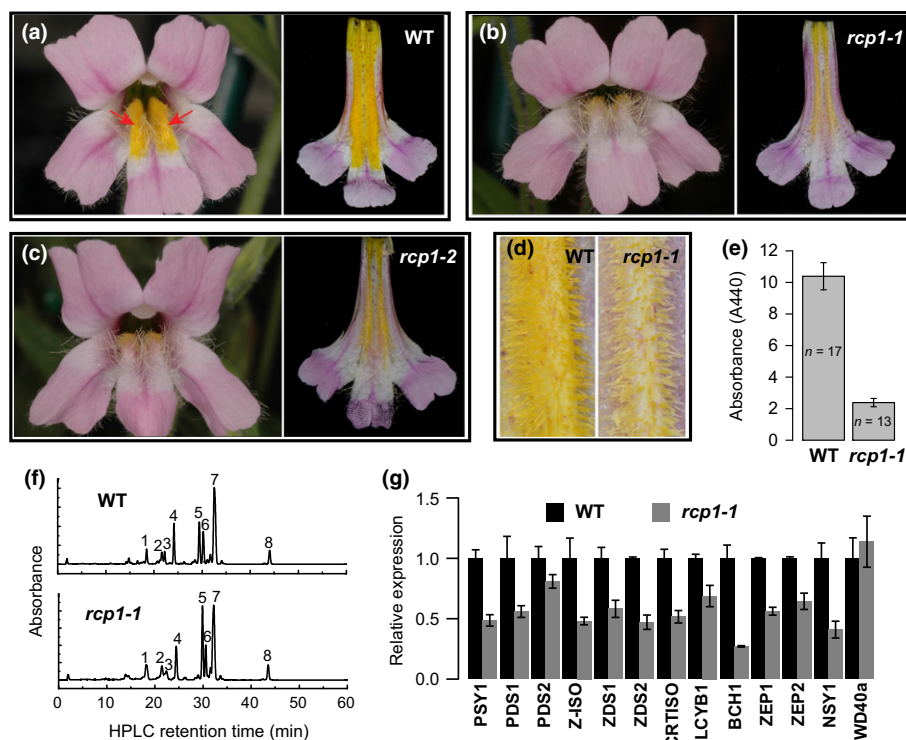


Fig. 2 *rcp1* is likely to be a transcription factor mutant. (a) Phenotype of wild-type (WT) *Mimulus lewisii* LF10 flower. Shown on the left is the front view of the corolla, with the yellow nectar guides marked by the red arrows; on the right is the dissected corolla displaying the complete nectar guides. (b, c) Phenotypes of the *rcp1-1* and *rcp1-2* mutants, shown in the same fashion as the WT. (d) Close-up of the trichomes in the middle region of the nectar guides; *rcp1-1* has normal trichomes. Note that the longer trichomes around the corolla throats are also visible in the front view of the corollas in (a–c). (e) Carotenoid concentration in the nectar guides, as estimated by absorbance measurement at 440 nm. Sample sizes are shown in the bar. Error bars are ± 1 SD. (f) High-performance liquid chromatography (HPLC) chromatograms of nectar guide carotenoids. The WT data were generated in a previous study (LaFountain *et al.*, 2015; reprinted with permission from Elsevier). The *rcp1-1* data were generated in this study using the same HPLC protocol. The eight peaks correspond to five carotenoid pigments: 1 and 3 represent the all-*trans* and *cis*- isomers of antheraxanthin, respectively; 2 and 4, all-*trans* and *cis*-violaxanthin; 5 and 6, all-*trans* and *cis*-deepoxyneoxanthin; 7, neoxanthin; 8, mimulaxanthin. (g) Quantitative reverse transcriptase-PCR of the carotenoid biosynthetic genes in the nectar guides of WT and *rcp1-1* (15-mm corolla stage). *MIUBC* was used as the reference gene as in previous studies (Yuan *et al.*, 2013a,b, 2014). *WD40a* was used as an additional control as this gene is not expected to be involved in carotenoid pigmentation. Error bars represent ± 1 SD from three biological replicates.

Carotenoid concentration and composition

The nectar guides (i.e. the two yellow ridges of the ventral petal) of each flower were carefully removed and weighed. The tissue was ground in 400 μ l methanol in a 1.5 ml Eppendorf tube and then soaked in the dark for 1 h. Following centrifugation, the supernatant was transferred to a new tube and evaporated under vacuum. Dried carotenoid pigments were resuspended in 100 μ l methanol and concentration was estimated by absorbance measurement at 440 nm. To take into account the size variation between flowers (some overexpression lines have smaller flowers), we normalized the absorbance values to 100 mg nectar guide tissue by weight. To determine the carotenoid composition of *rcp1-1* and the transgenic lines, the same normal-phase, high-performance liquid chromatography (NP-HPLC) protocol was used as in a previous study where the wild-type carotenoid composition was characterized (LaFountain *et al.*, 2015).

Bulk segregant analysis of the *rcp1-1* mutant

Bulk segregant analysis essentially followed Yuan *et al.* (2013b). Briefly, an F₂ population was produced from crossing *rcp1-1* and

the mapping line SL9. DNA samples from 100 F₂ segregants displaying the mutant phenotype were pooled with equal representation. A small-insert library (*c.* 400 bp) was prepared for the pooled sample and 100 bp single-end reads were generated by an Illumina HiSeq 2000 (Illumina, San Diego, CA, USA). The resulting *c.* 200 million short reads (NCBI SRA BioProject: PRJNA280080) were mapped to the SL9 pseudoscaffolds, and the resulting raw SNPs were filtered by depth of coverage, tendency of clustering, and variant frequency (Yuan *et al.*, 2013b). The SL9 pseudoscaffolds were then scanned for regions enriched with homozygous SNPs to determine the candidate gene interval.

Qualitative and quantitative reverse transcriptase-PCR

RNA extraction and cDNA synthesis followed Yuan *et al.* (2013a). Most of the carotenoid biosynthetic genes have two paralogs (except *Z-ISO* and *CRTISO*) in the *M. lewisii* genome (GenBank accessions: KR053166–KR053181). To examine the expression dynamics of these genes, qualitative reverse transcriptase (RT)-PCR was performed in leaf and four corolla developmental stages (5, 10, 15, and 20 mm). The genes responsible for carotenoid biosynthesis in the corolla are expected to increase in

expression level with corolla development, as the yellow intensity of the nectar guides gradually increases with the size of the flower bud, until the corolla reaches 15 mm, when the yellow becomes conspicuous. Four of the genes (*PSY2*, *LCYB2*, *BCH2* and *NSY2*) did not follow this expression pattern (Supporting Information Fig. S1). A previous 15 mm corolla transcriptome analysis also showed that these four genes are expressed at negligible level compared with their paralogs (Yuan *et al.*, 2014; LaFountain *et al.*, 2015). As such, these four genes were not subjected to further quantitative (q)RT-PCR. *MIUBC* was used as a reference gene for all RT-PCR experiments. RT-PCR primers are available from Table S1.

All qRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad) in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Reactions were run with three biological replicates and a single technical replicate. Samples were amplified for 40 cycles of 95°C for 15 s and 60°C for 30 s. Amplification efficiencies for each primer pair (listed in Table S1) were determined using critical threshold values obtained from a dilution series (1 : 4, 1 : 20, 1 : 100, 1 : 500) of pooled cDNAs. Relative expression of each target gene compared with the reference gene was calculated using the formula $(E_{\text{ref}})^{CP(\text{ref})}/(E_{\text{target}})^{CP(\text{target})}$ (Eqn 3 in Pfaffl, 2001) and standardized to the wild-type expression.

Plasmid construction and plant transformation

To build the RNAi construct, a 207 bp fragment from the first exon of the *RCPI* gene (nucleotide 92–298 from the translation initiation site) was cloned into the vector pFGC5941 (Kerschen *et al.*, 2004) in both sense and antisense orientation, as previously described (Yuan *et al.*, 2013a). This 207 bp fragment was chosen to maximize target specificity – no other genomic regions perfectly match this fragment for a contiguous block longer than 16 bp, as determined by BLASTing against the LF10 genome assembly with an *E*-value cutoff of 0.1.

To generate the overexpression plasmid, the full-length *RCPI* CDS (without the stop codon) was first cloned into the pENTR/D-TOPO vector (Invitrogen), then a linear fragment containing the *RCPI* CDS flanked by the attL1 and attL2 sites was amplified using M13 primers. This linear fragment was subsequently recombined into the Gateway vector pEarleyGate 103 (Earley *et al.*, 2006), which drives the expression of the transgene by the CaMV 35S promoter. The final plasmid constructs were verified by sequencing and then transformed into *Agrobacterium tumefaciens* strain GV3101 for subsequent plant transformation, as described in Yuan *et al.* (2013a). Primers used for amplifying cDNA fragments to construct the plasmids are listed in Table S2.

Results

rcp1 is likely to be a transcription factor mutant

To identify TFs regulating carotenoid biosynthesis during flower development, we performed an EMS mutagenesis experiment using the *M. lewisii* inbred line LF10, and recovered several fully recessive mutants with reduced carotenoid content in the nectar

guides (but without other obvious phenotypic alternations). Two of the mutants have identical phenotypes (Fig. 2b,c) and belong to the same complementation group, *reduced carotenoid pigmentation 1* (*rcp1*; the two allelic mutants will hereafter be referred to as *rcp1-1* and *rcp1-2*). Carotenoid concentration of the nectar guides is c. 4.4-fold lower in *rcp1* than in the wild-type (Fig. 2e).

Two lines of evidence suggest that *RCPI* encodes a transcriptional regulator. In the first, HPLC analyses revealed the same carotenoid composition for the wild-type and *rcp1* nectar guides, which consists of five carotenoid pigments with a mole percentage of antheraxanthin : violaxanthin : neoxanthin : deepoxyneoxanthin : mimulaxanthin = 10 : 20 : 45 : 20 : 5 (LaFountain *et al.*, 2015; Fig. 2f). This is best explained by a regulatory gene mutation, as a structural gene mutation is expected to change the carotenoid composition by eliminating products downstream of the mutated enzyme, as shown in *Arabidopsis* and tomato mutants (Ronen *et al.*, 2000; Isaacson *et al.*, 2002; Tian *et al.*, 2004; Kim & DellaPenna, 2006; North *et al.*, 2007). In the second, qRT-PCR showed coordinated transcriptional down-regulation of the entire CBP in *rcp1* (Fig. 2g). Most of the CBP structural genes showed 1.5- to 2.5-fold decrease in expression, with *BCH1* showing the strongest effect, an approximately four-fold decrease.

An *R2R3-MYB* is a strong candidate gene for *RCPI*

To genetically map *RCPI*, we carried out a bulk segregant analysis of the *rcp1-1* mutant by deep sequencing (see the Materials and Methods section). Intriguingly, *RCPI* was mapped to the same nine-gene interval (Fig. 3a,b) as a previously characterized gene, *GUIDELESS* (Yuan *et al.*, 2013b), which is required for trichome development in the nectar guides. However, complementation crosses suggested that these two are different genes, which was further supported by the observations that the *rcp1-1* mutant has normal trichomes in the nectar guides (Fig. 2d) and does not contain any mutations in the *GUIDELESS* gene. Examination of the mutant sequencing reads mapped to the other eight genes revealed a mutation resulting in a premature stop codon in the first exon of the mgv1a009251m ortholog (Fig. 3c), which encodes a subgroup 21 R2R3-MYB TF (GenBank KR053165), defined by the signature motif 'FxDFL' in the C-terminus of the protein (Fig. S2; Stracke *et al.*, 2001). The most closely related homologs of this gene in *Arabidopsis* are *AtMYB105/LOF2* and *AtMYB117/LOF1* (Fig. S3), which control lateral organ separation and axillary meristem formation (Lee *et al.*, 2009). Sequencing the cDNA of this gene in the allelic mutant *rcp1-2* showed a T-to-A mutation in an exon–intron junction that leads to nonsplicing of the first intron (Fig. 3c), which in turn results in several premature stop codons in the cDNA. These results suggest that the *R2R3-MYB* is most likely the causal gene underlying the *rcp1* mutants.

The *RCPI* candidate gene expression precedes the CBP structural genes

To examine the expression dynamics of this *R2R3-MYB* in the corolla, we performed RT-PCR across four developmental stages

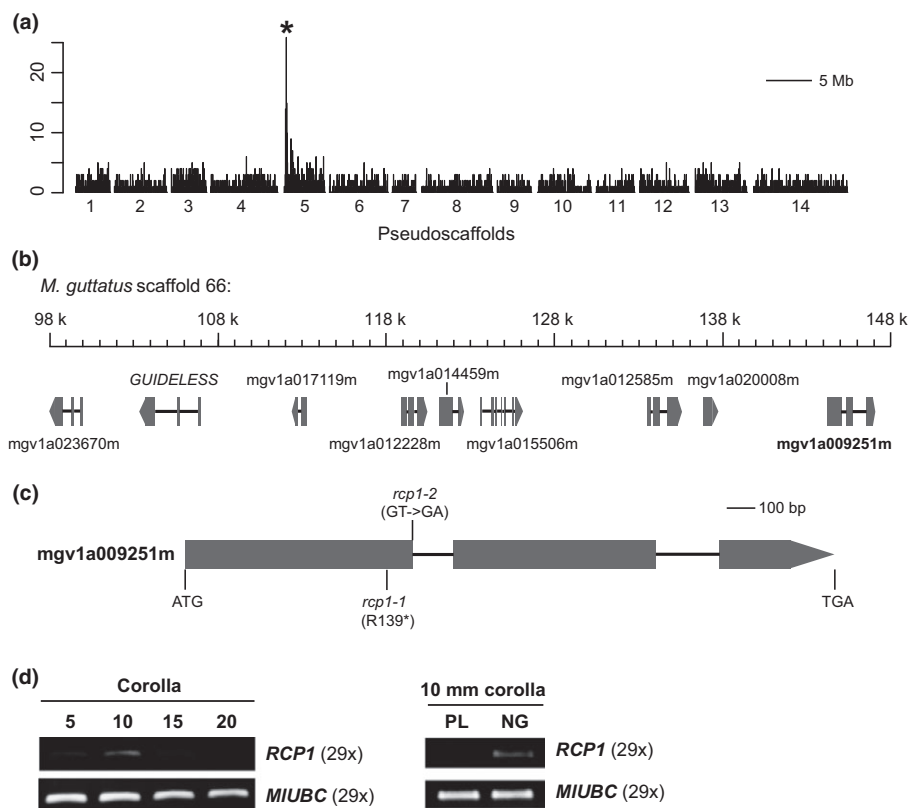


Fig. 3 An *R2R3-MYB* is a strong candidate gene for *Mimulus lewisii* *RCP1*. (a) Genome scan for regions that are enriched in homozygous single nucleotide polymorphisms (SNPs) revealed a sharp peak (asterisk). Each bar represents the number of homozygous SNPs in a 20 kb window. (b) The peak corresponds to a nine-gene interval. (c) One of the genes, *mgv1a009251m*, encodes an *R2R3-MYB* transcription factor and contains a premature stop codon in *rcp1-1* and a mutation at an exon–intron junction in *rcp1-2*. (d) Reverse transcriptase-PCR of the candidate *R2R3-MYB* gene across corolla developmental stages shows peak expression level at the 10 mm stage and its expression restricted to the nectar guides. *MIUBC* was used as the reference gene. PL, petal lobe; NG, nectar guides.

(Fig. 3d). Its expression increases from 5 mm (8 d before anthesis, DBA) to 10 mm (6 DBA), then decreases substantially from 10 to 15 mm (3 DBA) and becomes undetectable at 20 mm (1 DBA) (corolla developmental stages as described in Yuan *et al.*, 2013a). This expression pattern is consistent with the expected behavior of a transcriptional activator of the CBP structural genes: the *R2R3-MYB* expression precedes the structural genes, most of which reach their peak expression levels at 15 or 20 mm (Fig. S1). Dissection of the corolla into petal lobe and nectar guide tissue further revealed that the candidate gene is only expressed in the nectar guides (Fig. 3d).

Down-regulation of *RCP1* in the wild-type by RNAi phenocopies the mutant

To confirm that the candidate *R2R3-MYB* is in fact *RCP1*, we performed two transgenic experiments: knocking down the expression of this gene in the wild-type by RNAi was expected to recapitulate the *rcp1* phenotype, and overexpressing this gene in the *rcp1* mutant background should restore the yellow pigments in the nectar guides.

An RNAi plasmid was constructed with a 207 bp fragment from the first exon of the candidate *R2R3-MYB* gene and was transformed into the wild-type LF10. This 207 bp fragment was chosen to minimize the off-target effect: no other genomic regions perfectly match this fragment for a contiguous block longer than 16 bp, as determined by BLASTing against the LF10 genome assembly with an *E*-value cutoff of 0.1. We obtained four independent stable RNAi transgenic lines with phenotypes

indistinguishable from the *rcp1* mutants (Figs 4a,b, S4a). The total carotenoid concentration of the nectar guides is 3.8- to 4.4-fold lower in the RNAi lines than in the wild-type (Figs 4b, S4a), and the carotenoid composition remains the same (Fig. S5). qRT-PCR showed an *c.* 80% knockdown of the candidate *MYB* gene in the RNAi lines (Figs 4c, S4b). We also examined the expression level of five structural genes that showed twofold or greater down-regulation in the *rcp1-1* mutant, and found a similar down-regulation of all these genes in RNAi line-1 (Fig. 4d).

Overexpressing *RCP1* in the *rcp1* mutant background restores carotenoid production

An overexpression construct that contained the full-length CDS of the *MYB* gene driven by the CaMV 35S promoter was transformed into *rcp1-1*. We obtained 78 independent overexpression (OE) lines in which the carotenoid concentration was completely or largely restored (Fig. 4b,e), with the carotenoid composition remaining the same (Fig. S5). The phenotypes of these OE lines fall into four major types, represented by lines 2, 85, 80 and 8 (Fig. 4e). The transgene expression levels in these four representative lines are approximately five-, 15-, 47- and 93-fold higher, respectively, than the endogenous *RCP1* peak expression level in the wild-type nectar guides (6 DBA) (Fig. 4f).

The OE plants represented by OE-2 are the most similar to the wild-type. As the transgene expression level increases (as in OE-85 and OE-80), the nectar guide carotenoid concentration increases only slightly (Fig. 4b), but the petal lobe becomes white, owing to a dramatic decrease in anthocyanin production, and the

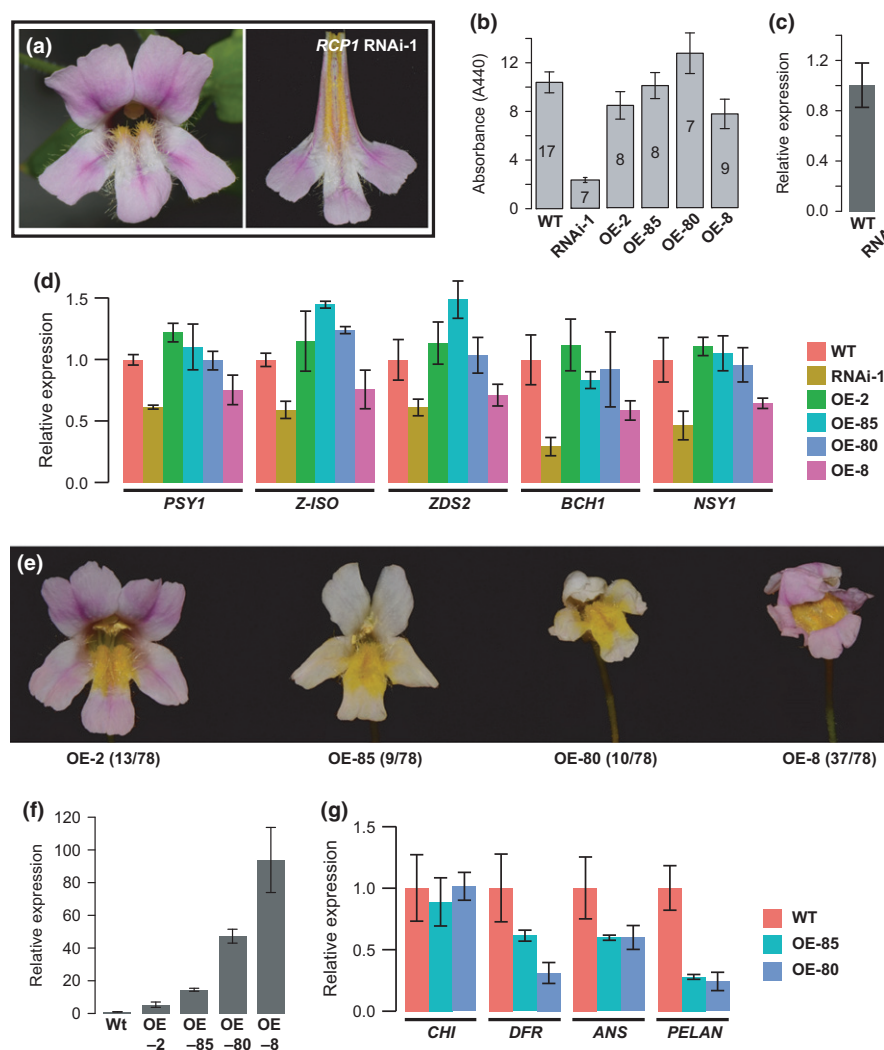


Fig. 4 Transgenic experiments verify *RCP1* function in *Mimulus lewisii*. (a) The phenotype of *RCP1* RNAi lines (in the wild-type (WT) background) is indistinguishable from *rcp1* mutants. (b) Carotenoid concentration of the transgenic lines, estimated by absorbance measurement at 440 nm. Sample sizes are shown in the bar. Error bars are ± 1 SD. (c) Relative transcript abundance of *RCP1* in 10 mm nectar guides of *RCP1* RNAi line-1 as measured by quantitative reverse transcriptase (qRT)-PCR. (d) The relative transcript abundances of five selected carotenoid biosynthetic genes in the transgenic lines. (e) Phenotypes of the 78 *RCP1* overexpression (OE) lines (in the *rcp1-1* background) fall into four major types, represented by lines 2, 85, 80 and 8, respectively. The proportion of each type is shown after the representative line number, and the remaining nine lines have intermediate phenotypes between the major types. (f) The transgene transcript abundances of the four representative lines are approximately five, 15, 47 and 93-fold higher than the endogenous *RCP1* peak expression level. (g) Relative transcript abundances of three anthocyanin biosynthetic genes (*CHI*, *DFR*, *ANS*) and the anthocyanin-activating MYB (*PELAN*) in the white-flowered OE lines. *MYB* was used as the reference gene for all qRT-PCR experiments. Error bars in all gene expression graphs represent ± 1 SD from three biological replicates.

flowers become smaller in size (Fig. 4e). However, when transgene expression exceeds a certain level (as in OE-8), the phenotype becomes weaker: the nectar guide carotenoid concentration is slightly lower than in the wild-type, and the petal lobes are pink (albeit more folded and twisted than the wild-type) (Fig. 4b,e). One possible explanation for this inverse relationship between transgene expression level and phenotypic effect in OE-8 is protein aggregation induced by high protein concentration (Zettlmeissl *et al.*, 1979; Bire *et al.*, 2013). Consistent with the carotenoid phenotype, expression levels of the CBP structural genes are restored to wild-type level (or slightly higher than the wild-type level) in the OE plants, except in OE-8 (Fig. 4d), in which the structural gene expression levels are only partially restored.

Overexpressing *RCP1* decreases anthocyanin production in some transgenic lines

Unexpectedly, *RCP1* appears to negatively regulate anthocyanin production in the petal lobe when expressed at moderately high levels (as in OE-85 and OE-80; Fig. 4e). The anthocyanin

biosynthetic pathway contains at least six essential structural genes: *Chalcone synthase* (*CHS*), *Chalcone isomerase* (*CHI*), *Flavonoid 3-hydroxylase* (*F3H*), *Dihydroflavonol 4-reductase* (*DFR*), *Anthocyanidin synthase* (*ANS*), and *UDP-3-O-glucosyltransferases* (*UGT*). The 'late' pathway genes (*F3H*, *DFR*, *ANS*, and *UGT*) were shown to be coordinately activated by a highly conserved MYB-bHLH-WD40 protein complex in *M. lewisii* (Yuan *et al.*, 2014), whereas the 'early' pathway genes (*CHS* and *CHI*) are regulated by unknown factors. To understand the mechanism underlying the negative regulation of anthocyanin biosynthesis by overexpressed *RCP1*, we performed qRT-PCR of three anthocyanin biosynthetic genes (*CHI*, *DFR*, and *ANS*) that represent both the 'early' and 'late' pathways in OE-85, OE-80, and wild-type petal lobes. While *CHI* did not show any expression change in the white-flowered OE lines compared with the wild-type, both *DFR* and *ANS* were down-regulated approximately two- to threefold in the OE lines (Fig. 4g).

The coordinated down-regulation of the 'late' pathway genes implies a down-regulation of the MYB-bHLH-WD40 regulatory complex. Our previous work has shown that down-

regulation of the *bHLH* or *WD40* gene reduces anthocyanin production in multiple tissues (Yuan *et al.*, 2014), but the *RCPI* OE lines only show anthocyanin reduction in the petal lobe. Therefore, we hypothesized that the petal lobe-specific *MYB* TF, *PELAN* (Yuan *et al.*, 2014), is down-regulated by the overexpression of *RCPI*. qRT-PCR experiments clearly showed that this is the case: *PELAN* expression is approximately four-fold lower in OE-85 and OE-80 petal lobes than in the wild-type (Fig. 4g). These results indicate that there is potential crosstalk between the regulatory networks underlying the two major pigment types.

Discussion

Identification of TFs underlying floral carotenoid pigmentation has remained elusive, largely because the conventional flower genetic model systems (e.g. *Arabidopsis*, *Antirrhinum*) do not accumulate carotenoids in their flowers. In this study we have analyzed a regulatory gene mutant of *M. lewisii* with reduced carotenoid pigmentation in the flower nectar guides. Based on evidence from genetic mapping, two independent mutant alleles, and transgenic experiments, we identified an R2R3-MYB TF, *RCPI*, as a master regulator that controls the entire CBP during flower development. Our results demonstrate that *M. lewisii* is a powerful new genetic model system, particularly suitable to study the developmental genetics of important floral traits that are difficult or unfeasible to study using previously well-established systems.

RCPI belongs to the R2R3-MYB subgroup 21, which is readily recognized by the signature motif 'FxDFL' in the C-terminus of the protein (Fig. S2; Stracke *et al.*, 2001) and is clearly distinguishable from the R2R3-MYBs that activate anthocyanin or flavonol biosynthesis (i.e. subgroup 6 and 7 in Fig. S3; Dubos *et al.*, 2010). Subgroup 21 contains seven related genes in *Arabidopsis* (Fig. S2), with a wide range of regulatory functions in secondary cell wall thickening, lateral organ separation, axillary meristem formation, and ABA response (Zhong *et al.*, 2008; Lee *et al.*, 2009; Park *et al.*, 2011). But little is known about the function of these MYBs in other plants. Our results show that they are also involved in the regulation of pigment production. This heterogeneity of gene function is in sharp contrast to other R2R3-MYB subgroups. For example, all subgroup-6 MYBs characterized to date (including *PELAN* and *NEGAN* in *Mimulus*; Yuan *et al.*, 2014) seem to regulate anthocyanin biosynthesis, and all subgroup-9 MYBs (including *GUIDELESS* in *Mimulus*; Yuan *et al.*, 2013b) regulate trichome and conical cell development. How do these similar MYBs belonging to subgroup 21 perform so many seemingly unrelated functions? One possibility is that these MYBs interact with different partners in different developmental contexts. The different protein complexes, instead of the MYB alone, determine the target gene specificity. Work to identify potential interacting partners of *RCPI* is currently under way in our laboratory.

Although *RCPI* activates carotenoid production in the nectar guides, ectopic expression of *RCPI* does not lead to carotenoid accumulation in the petal lobe. This can be explained by at least

two nonexclusive possibilities: the dominant repressor *YELLOW UPPER* (*YUP*), which is known to prevent carotenoid deposition in the wild-type petal lobes (Hiesey *et al.*, 1971; Bradshaw & Schemske, 2003), is epistatic to *RCPI*; and the function of *RCPI* is dependent on an interacting partner that is present in the nectar guides but absent from the petal lobes. Testing these possibilities will require identification of *YUP* and potential *RCPI*-interacting partners.

It is worth noting that *RCPI* can simultaneously activate carotenoid biosynthesis and repress anthocyanin production when expressed at moderately high levels. Previous studies on tomato *high pigment* mutants have shown that genes involved in light signal transduction can simultaneously repress anthocyanin production in vegetative tissues and repress carotenoid production in mature fruits (Mustilli *et al.*, 1999; Liu *et al.*, 2004). But there is no evidence for direct interactions of the two pigment pathways in the same tissue. Our results show that overexpressing *RCPI* can simultaneously activate carotenoid biosynthetic genes and repress the expression of an anthocyanin-activating *MYB* TF (i.e. *PELAN*) in the flower, thereby establishing a potential direct link between the two pigment pathways. This behavior of turning one pigment pathway on and simultaneously turning the other off is particularly intriguing in light of the observation that multiple species in the genus *Mimulus* display intraspecific flower color polymorphism (anthocyanin-based purple vs carotenoid-based yellow), including *M. mephiticus*, *M. parryi*, *M. whitneyi*, and *M. discolor* (Thompson, 2005; Fraga, 2012). The two color morphs are commonly mixed in natural populations and there are no intermediate phenotypes (Thompson, 2005; Fraga, 2012), which indicates a simple genetic switch between anthocyanin and carotenoid production. *RCPI* appears to display the properties of such a genetic switch. It will be interesting to test whether the *RCPI* ortholog in the aforementioned species plays a role in controlling color dimorphism.

Carotenoids provide distinct colors for a large number of flowers and fruits. The tremendous diversity of carotenoid pigmentation patterns among plant species is largely determined by when and where the carotenoid biosynthetic genes are expressed (i.e. the transcriptional regulation of the structural genes). As such, identification of TFs that regulate carotenoid biosynthesis in flowers or/and fruits, such as *RCPI*, is critical to understanding the molecular basis of flower and fruit color variation in nature. Moreover, carotenoids are essential components of human diets as precursors for vitamin A biosynthesis and as antioxidants for disease prevention (Fraser & Bramley, 2004; Farré *et al.*, 2011). Previous attempts to enhance carotenoid production in staple crops via genetic engineering have focused on overexpressing one or a few enzymes that are regarded as rate-limiting for carotenoid biosynthesis, as in 'Golden Rice' (Ye *et al.*, 2000) and tomatoes (Fraser *et al.*, 2002). These strategies often suffer from secondary restrictions caused by relatively low expression level of a downstream enzyme (Fraser & Bramley, 2004; Farré *et al.*, 2011). TFs such as *RCPI* can coordinately up-regulate the entire CBP and should be an obvious target for future genetic manipulation in crop plants.

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Author contributions

Y-W.Y. planned and designed the research. J.M.S., L.E.S., A.M.L., C.L. and Y-W.Y. performed experiments. J.M.S., L.E.S., A.M.L., H.A.F. and Y-W.Y. analyzed the data, and Y-W.Y. wrote the manuscript with input from all authors.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 RT-PCR of CBP structural gene paralogs.

Fig. S2 Alignment of RCP1 and the most closely related homologs in *Arabidopsis*.

Fig. S3 A maximum likelihood (ML) phylogeny of various R2R3-MYBs.

Fig. S4 Characterization of additional *RCP1* RNAi lines.

Fig. S5 HPLC chromatograms of carotenoids in the nectar guides of transgenic lines.

Table S1 Primers used in RT-PCR experiments

Table S2 Primers used for constructing RNAi and overexpression plasmids

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