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Carotenoid composition of the flowers of *Mimulus lewisii* and related species: Implications regarding the prevalence and origin of two unique, allenic pigments

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ABSTRACT

The genus *Mimulus* has been used as a model system in a wide range of ecological and evolutionary studies and contains many species with carotenoid pigmented flowers. However, the detailed carotenoid composition of these flowers has never been reported. In this paper the floral carotenoid composition of 11 *Mimulus* species are characterized using high-performance liquid chromatography, mass spectrometry and chemical methods with a particular focus on the genetic model species, *Mimulus lewisii*. *M. lewisii* flowers have five major carotenoids: antheraxanthin, violaxanthin, neoxanthin, and the unique allenic carotenoids, deepoxyneoxanthin and mimulaxanthin. This carotenoid profile is consistent with the expression levels of putative carotenoid biosynthetic genes in the *M. lewisii* flower. The other 10 species possess the same five carotenoids or a subset of these. Comparison of the carotenoid profiles among species in a phylogenetic context provides new insights into the biosynthesis and evolution of deepoxyneoxanthin and mimulaxanthin. This work also lays the foundation for future studies regarding transcriptional control of the carotenoid biosynthesis pathway in *Mimulus* flowers.

Introduction

The wildflower genus *Mimulus* (Monkeyflowers) contains 160–200 species with tremendous phenotypic variation [1] (Fig. 1) and has served as a classic model system in studying ecological adaptation [2–6], speciation [2,7–10] and plant–pollinator interactions [11–14]. More recently, it has also attracted considerable interest in studies of meiotic drive [15], population genomics [16,17], and evolution of development (Evo-Devo)[1] [18,19]. In particular, the species *Mimulus lewisii* is emerging as an excellent system to elucidate the genes and developmental pathways underlying ecologically important floral traits, largely due to the recent development of genomic resources that enable rapid genetic mapping, a collection of chemically induced mutants showing remarkable floral trait alterations, and a powerful stable transformation protocol that allows rigorous characterization of gene function by transgenic experiments [10,14,19,20].

Carotenoid pigmentation is an ecologically important yet genetically poorly understood floral trait. Many closely related plant species display dramatically different carotenoid pigmentation patterns in their flowers [21], and in some cases the pattern change has produced a pollinator shift leading to reproductive isolation and speciation [13]. In vegetative tissues carotenoids are essential components of the photosynthesis system: they act as accessory pigments in light-harvesting and play an indispensible role in protecting the photosynthetic apparatus from photooxidative damage [22–26]. All plants contain a functional set of structural genes for carotenoid biosynthesis that have been well-characterized in multiple plant systems [27,28] (Fig. 2). The diversity of carotenoid pigmentation patterns in angiosperm flowers is determined largely by when and where these structural genes are expressed; i.e., transcriptional regulation of the structural genes [21]. Yet the transcriptional regulation of carotenoid biosynthetic genes remains the largest gap in the current understanding of floral carotenoid pigmentation. With the remarkable natural variation between closely related species (Fig. 1) and a number of chemically-induced mutants that affect transcriptional control of carotenoid accumulation in the yellow nectar guides of *M. lewisii*

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[1] *Abbreviations used:* Evo-Devo, evolution of development; NMR, nuclear magnetic resonance; IR, infrared; NP, normal-phase; HPLC, high performance liquid chromatography; APCI, atmospheric pressure chemical ionization; LCYE, lycopene ε-cyclase; LCYB, lycopene β-cyclase; NSY, neoxanthin synthase enzyme; $LiAlH_4$, lithium aluminum hydride.

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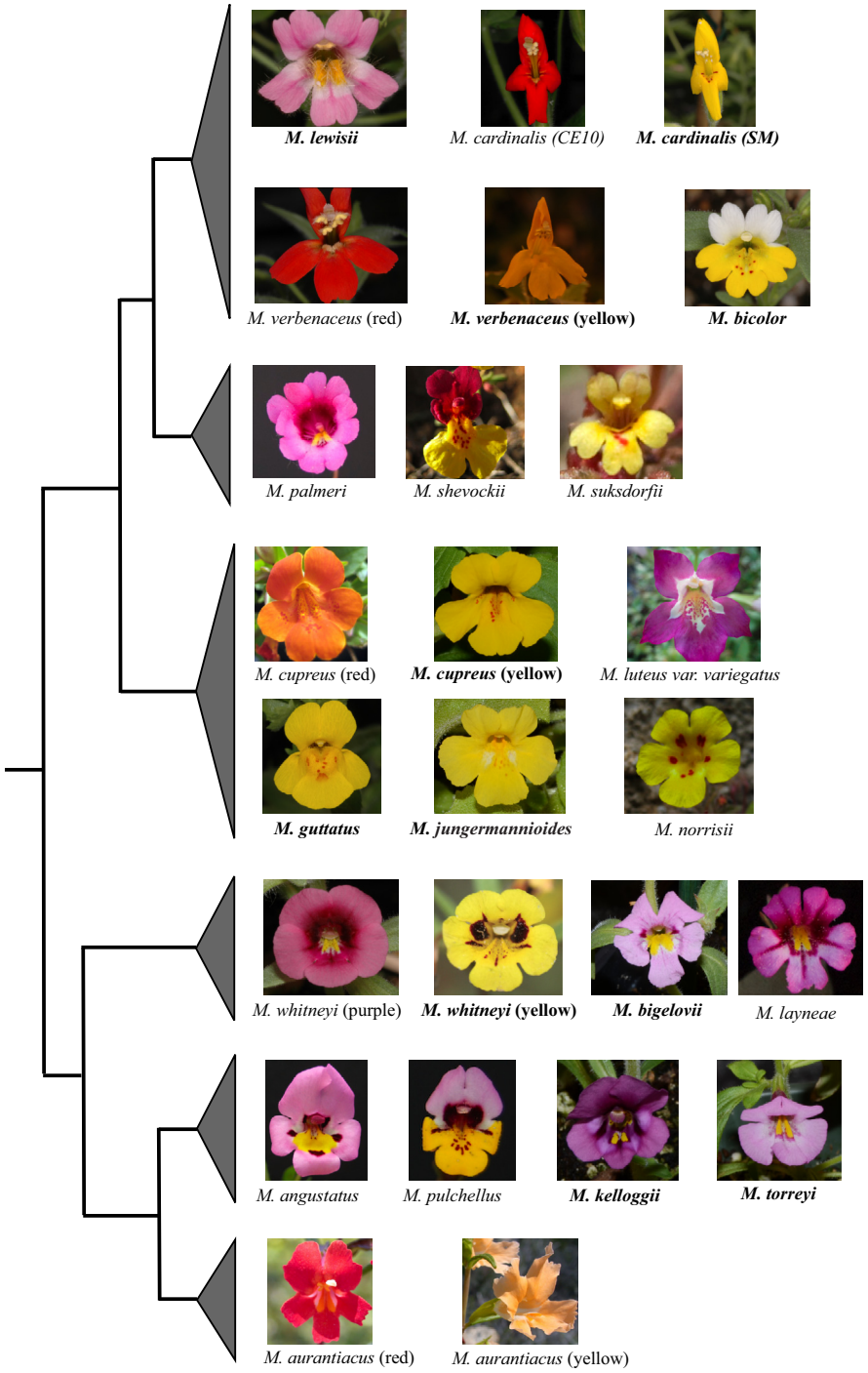

Fig. 1. Natural carotenoid pigmentation variation among *Mimulus* species. Shown on the left is a schematic illustration of the phylogenetic relationships among the six major *Mimulus* clades, based on Beardsley et al. [1] and Grossenbacher and Whittall [51]; on the right are representative species of each clade. Images of *M. palmeri*, *M. shevockii*, *M. suksdorfii*, *M. norrisii*, *M. layneae*, *M. angustatus* and *M. pulchellus* were provided by Dena Grossenbacher; images of *M. aurantiacus* were provided by Matt Streisfeld; the remaining images were taken by Y-W.Y. Some species have yellow all over the corolla (e.g., *M. guttatus*) whereas others have carotenoids only in the two yellow stripes (i.e. nectar guides) on the lower petal (e.g., *M. lewisii*). Species with names highlighted in bold were analyzed in this study. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Yuan, unpublished data), *Mimulus* holds great promise to help fill this knowledge gap.

A foundational step towards the systematic investigation of the transcriptional control of floral carotenoid pigmentation is a characterization of the carotenoid composition in the flowers of representative species, especially the genetic model species, *M. lewisii*. Only a few *Mimulus* species have been analyzed for their floral carotenoid composition [29–32]. These studies were conducted more than 40 years ago and some of the characterizations were inconclusive. In the first report of *Mimulus* carotenoid composition, which was conducted on the flowers of *Mimulus longiflorus*, Zechmeister and Schroeder [29] reported that the orange petals were colored by the open-chain carotenoids, γ-carotene and lycopene (Fig. 2), but noted a great deal of individual

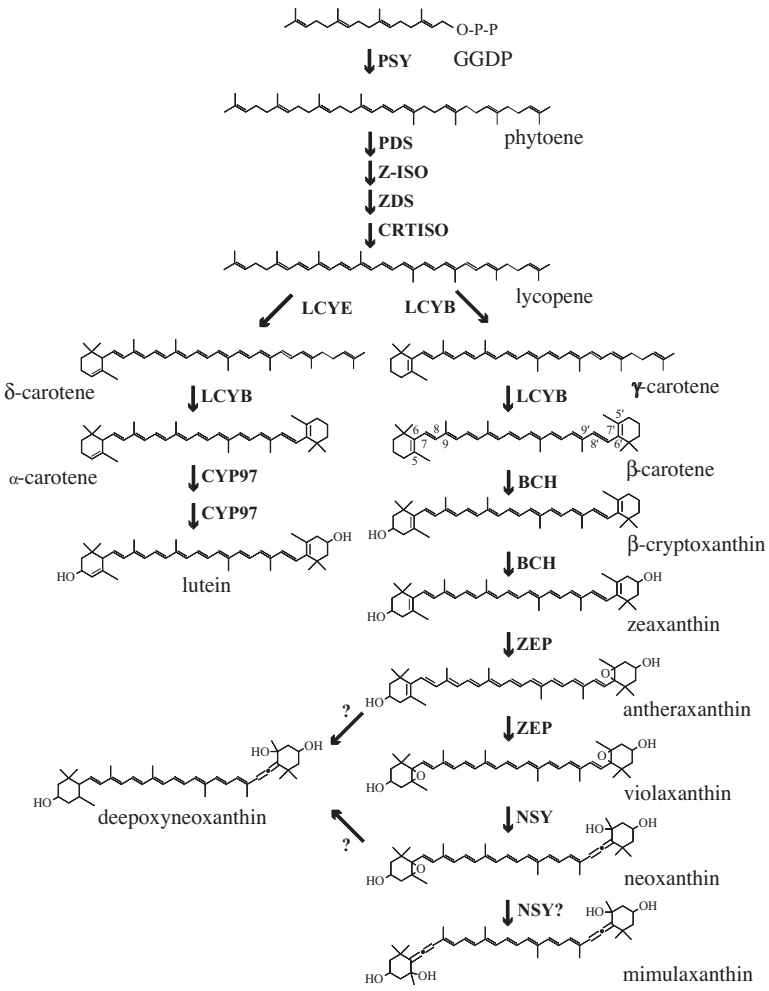

Fig. 2. Chemical structures of carotenoids and the carotenoid biosynthesis pathway (modified from [28]). GGDP, geranylgeranyl diphosphate; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, 15-*cis*-ζ-carotene isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LCYE, lycopene ε-cyclase; LCYB, lycopene β-cyclase; BCH, β-carotenoid hydroxylase; ZEP, zeaxanthin epoxidase; NSY, neoxanthin synthase. The IUPAC numbering scheme [41] is indicated for some of the carbons in β-carotene.

variation in the relative concentrations of these pigments. Some samples also were reported to contain the cyclic carotenoids, zeaxanthin and cryptoxanthin [29].

Goodwin and Thomas [30] analyzed the carotenoid composition of the petals of *Mimulus cupreus* (red variety) and *Mimulus tigrinus* (a hybrid cultivar with unclear origin). They reported *M. cupreus* to contain primarily the carotenoid β-carotene (>80%, Fig. 2) and three xanthophylls, one of which was tentatively identified as lutein epoxide (therein referred to as "taraxanthin"). *M. tigrinus* was reported to contain nine carotenoids, the most abundant of which was identified as lutein epoxide. This conclusion was based on the similarities in the absorption spectrum and chromatographic behavior of the pigment with those of an authentic lutein epoxide sample that was isolated and purified from the flowers of *Taraxacum officinale* [30]. However, a later study [33] using mass spectrometry revealed that the pigment identified as lutein epoxide from *Taraxacum* was in fact all-*trans* neoxanthin.

Nitsche and colleagues [31,34] investigated the yellow pigments of *Mimulus guttatus* petals in an attempt to determine the unidentified xanthophylls reported by Goodwin and Thomas [30]. They reported that the petals contained neoxanthin and two neoxanthin-derived allenic carotenoids, deepoxyneoxanthin and mimulaxanthin (Fig. 2). Subsequent data from mass spectrometry, nuclear magnetic resonance (NMR) and infrared (IR) spectroscopy allowed Nitsche [32] to update the structural assignment of mimulaxanthin as 6,7,6′,7′-tetradehydro-5,6,5′,6′-tetrahydro,β,β-carotene-3,5,3′,5′-tetrol, which is a symmetric molecule with two allenes and four hydroxyls (Fig. 2). This remains the currently accepted structure of mimulaxanthin [35,36].

The present work seeks to elucidate comprehensively the carotenoid composition of several species representing the major evolutionary lineages of *Mimulus*, with a particular emphasis on *M. lewisii*, and to examine the variation of carotenoid composition among species in a phylogenetic context to gain insights into the biosynthesis and evolution of deepoxyneoxanthin and mimulaxanthin.

Methods

Collection of plant tissues

Flowers were collected from 11 species cultivated in the greenhouse at the Department of Ecology and Evolutionary Biology at the University of Connecticut: *Mimulus cupreus* (yellow form, [18]), *Mimulus guttatus*, *Mimulus jungermannioides*, *M. lewisii* (inbred line LF10), *Mimulus cardinalis* (inbred line SM, [19]), *Mimulus verbenaceus* (yellow form, [11]), *Mimulus bicolor*, *Mimulus whitneyi* (yellow form), *Mimulus bigelovii*, *Mimulus kelloggii* and *Mimulus torreyi*. These species represent four of the six major evolutionary lineages of *Mimulus* (Fig. 1). Care was taken to remove the anthers and non-carotenoid containing portions of the flower by trimming them away with scissors. Immediately

after collection, the petals were placed in a −20° freezer where they were stored until pigment extraction.

Pigment extraction and preparation

In order to extract the carotenoids, the petals were transferred to a 15 mL glass Potter–Elvehjem tissue homogenizer into which 5 mL of methanol was added to cover the petals. The sample was then ground manually for 3–5 min. The solvent containing the pigments was carefully pipetted out of the homogenizer, fresh methanol was added to the homogenizer, and the manual grinding process was repeated to ensure complete recovery of pigments. This procedure was continued until the petals appeared colorless, which typically took three or four repetitions.

The resulting solution was then placed into 1.5 mL Eppendorf tubes, loaded into a Fisher Scientific 235C bench-top centrifuge and spun at 13,600×*g* for 2 min. Following centrifugation, the supernatant from each tube was carefully pipetted out, combined, and dried under nitrogen gas. The sample was then subjected to saponification using ethanolic KOH according to the method described by Schiedt and Liaaen-Jensen [37]. Briefly, each sample was dissolved in a mixture consisting of 1 mL of diethyl ether and 1 mL of 2.5% ethanolic KOH solution (50% (w/v) KOH solution in water, diluted 1:9 with ethanol) [37]. The sample vial was then capped and kept at room temperature in the dark for two hours. Subsequently, the sample was transferred to a 12.5 mL glass vial containing 4 mL of 1:1 hexane/diethyl ether to which 5 mL water was added. The sample was mixed thoroughly and the vial was then placed on ice to allow the solvent layers to separate. The bottom aqueous phase was removed using a pipet and the process of washing the organic phase with 5 mL of water was repeated 5–8 times to remove the KOH and until the aqueous phase gave a neutral reading on pH paper. The upper layer from each sample containing the carotenoid pigments was then evaporated to dryness under nitrogen gas. In order to prevent degradation during processing, the extraction procedure was conducted under low-light, and samples were kept on ice at all times, the exception being during saponification.

Structure elucidation

In preparation for analysis by normal-phase (NP) high performance liquid chromatography (HPLC), the KOH-treated sample was dissolved in 14% acetone in hexane and filtered using a Millex 0.2 μm syringe-driven filter unit. The sample was then injected into a Waters 600 HPLC system employing a 2996 photodiode array detector. A Waters Sunfire analytical silica column (5 μm, 4.6 mm × 250 mm) was used for the initial evaluation of the sample. Subsequently, a Waters Sunfire preparative silica OBD column (5 μm, 19 mm × 100 mm) was used to collect the individual pigments from *M. lewisii* and *M. verbenaceus* for mass spectrometry and chemical analysis. The mobile phase for both columns consisted of: solvent A, hexane; and solvent B, acetone. The program began with 90% solvent A and 10% solvent B and was adjusted linearly over a period of 60 min to a final composition of 50% solvent A and 50% solvent B, which was then delivered isocratically for the remainder of the run. The flow rate was 2.0 mL/min using the analytical column and 7.0 mL/min for the preparative column. After collecting the pigments from the HPLC, they were dried under nitrogen gas and stored at −20 °C until needed for further analysis.

Synthetic mimulaxanthin [36] was obtained as a gift from Dr. Shigeo Katsumura (Osaka City University) and chromatographed using the same NP-HPLC protocol for direct comparison of the retention times and absorption spectra in the mobile phase solvent with the carotenoids observed from the flower petals. In lieu of

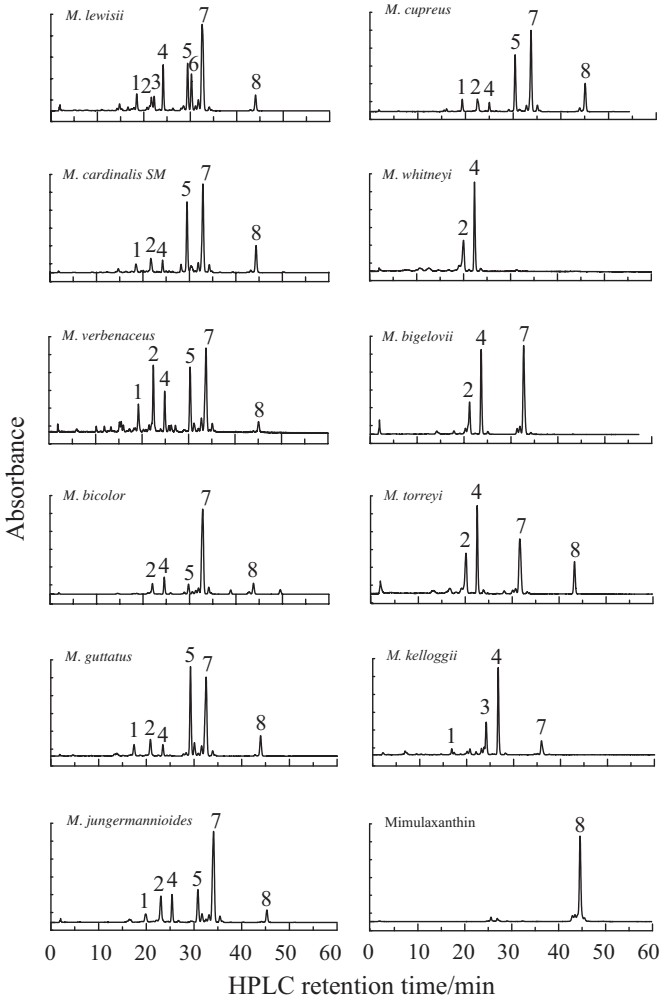

Fig. 3. HPLC chromatograms of extracts from *Mimulus* petals, along with a synthetic mimulaxanthin sample. All chromatograms are detected at 450 nm. Pigments are denoted numerically as follows: 1, all-*trans* antheraxanthin; 2, all-*trans* violaxanthin; 3, *cis*-antheraxanthin; 4, *cis*-violaxanthin; 5, deepoxyneoxanthin; 6, *cis*-deepoxyneoxanthin; 7, neoxanthin (all-*trans* and/or *cis*); 8, mimulaxanthin.

bona fide standards of other xanthophylls, pigment extract from dark-adapted *Spinacia oleracea* (spinach) which has been well-characterized and found to contain the carotenoids neoxanthin, violaxanthin, lutein, and β-carotene [38–40], was prepared by soaking leaves in methanol, centrifuging the extract at 13,600×*g* for 2 min as described above and drying the supernatant under nitrogen. This extract was chromatographed using the same NP-HPLC protocol as was used for the flower petal extracts to aid in the tentative identifications of these xanthophylls.

The pigments obtained from the preparative HPLC run were analyzed by mass spectrometry using an Applied Biosystems API 2000 system equipped with an atmospheric pressure chemical ionization (APCI) probe. The samples were dissolved in acetonitrile and injected using the following parameters: probe temperature, 400 °C; nebulizer current, 2 μA; curtain gas, 20.0 psi; ion source gas 1, 20.0 psi; ion source gas 2, 6.0 psi; interface heater, on; declustering potential, 60.0 V; focusing potential, 320 V. The mobile phase was acetonitrile delivered at a rate of 100 μL/min.

The number of epoxide groups present in each of the HPLC-purified pigments was determined by a confirmatory test using formic acid, as described by Schiedt and Liaaen-Jensen [37]. Formic acid causes the C(5,6) epoxide group to rearrange into a C(5,8) epoxide (see the IUPAC carbon numbering scheme [41] on β-carotene in Fig. 2). This rearrangement results in the removal of one double

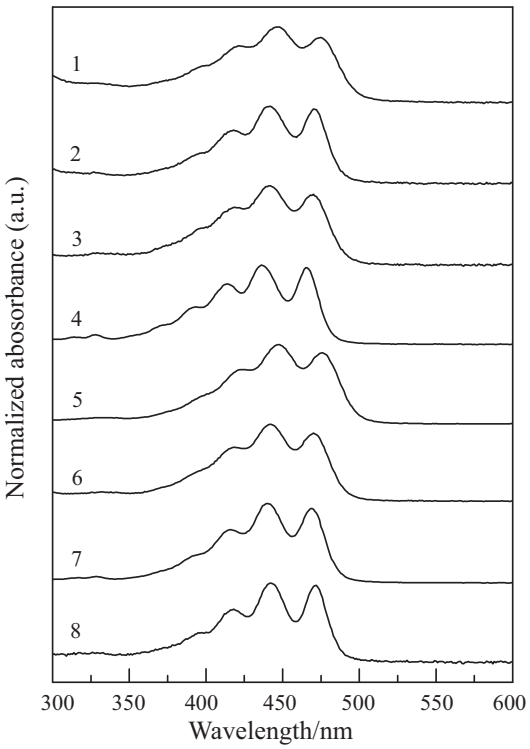

Fig. 4. Absorption spectra of pigments in ethanol. Numbers correspond to labels used in Fig. 3.

bond from the conjugated chain and is indicated by an ~20 nm blue-shift of the absorption spectrum per epoxide. Pigments collected from multiple separations of *M. lewisii* extract were combined and dissolved in ethanol, and the absorption spectrum of the sample was recorded using a Varian Cary 50 UV/vis spectrophotometer. Twenty drops of formic acid was then added directly to the cuvette in two ten-drop increments. After each ten-drop addition, the sample was mixed thoroughly with a pipette, and the absorption spectrum was re-recorded.

Carotenoid biosynthetic gene expression

Expression levels of the putative carotenoid biosynthetic genes in the *M. lewisii* flower were estimated by the RPKM value [42] based on a previous transcriptome analysis [19].

Results

Analysis using an NP-HPLC protocol revealed that the nectar guides of *M. lewisii* (the two yellow stripes on the lower petal; Fig. 1) contain eight major saponified carotenoid pigments (Figs. 3 and 4), which together account for 99% of the carotenoid composition (Fig. 5 and Table S1). These pigments are relatively polar, as evidenced by their generally overall long (≥20 min) retention times on the silica column (Fig. 3). The other sampled *Mimulus* species have a subset of these 8 pigments (Fig. 3 and Table S1).

Chromatography of the spinach extract (Fig. S1) using the same separation protocol revealed two pigments with similar retention times to those obtained from the *Mimulus* flower petals, including violaxanthin, which eluted at 20 min and neoxanthin, which eluted at 32 min. It should be noted that neoxanthin from green leaves occurs primarily in the 9′-*cis* configuration [35,43], whereas neoxanthin from other plant parts such as flowers, seeds, or fruit may adopt either a 9′-*cis* or an all-*trans* configuration [43]. The two structures can be differentiated by an approximate 5 nm difference in the λ_{max} of their absorption spectra [43]. Chromatography of synthetic mimulaxanthin revealed a retention time of 45 min (Fig. 3).

Identifications of the pigments in the *Mimulus* samples were made on the basis of the comparison of the HPLC retention times with those of the control standards, and on comparison of the absorption spectra (Fig. 4) of the pigments with previously published data [35]. These identifications were further supported by mass spectrometry data and the epoxide confirmatory test (Table 1).

In order to simplify the presentation of the results of the HPLC analysis in Fig. 3, the individual peaks corresponding to specific carotenoids found in the flower petals are numbered 1–8 in order of retention time. It is important to note that a variation of between 1 to 3 min between runs in the retention times of the same eluting pigments is not uncommon for NP-HPLC protocols [44].

Peak 1, observed at 17–20 min (Fig. 3), was found to have a λ_{max} at 447 nm (Fig. 4) and one epoxide group. The mass of this pigment was found to be 584 *m*/*z*. These data are consistent with the carotenoid, antheraxanthin (Fig. 2), which is commonly found in yellow flowers [35,45].

Peak 2, observed at 20–23 min (Fig. 3), shows a λ_{max} at 442 nm (Fig. 4). Two epoxide groups were revealed from the chemical test, and mass spectral analysis indicated a mass of 600 *m*/*z*. These data are consistent with the all-*trans* form of violaxanthin [35]. In further support of this identification, the retention time of this

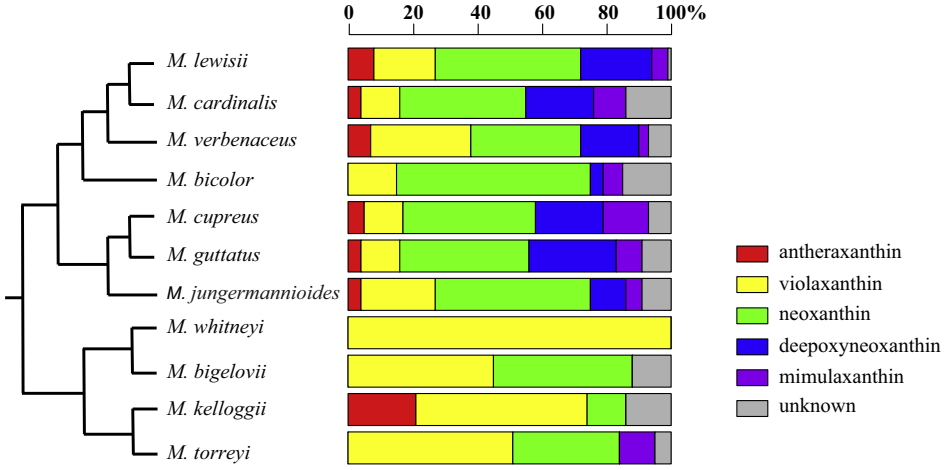

Fig. 5. Variation of carotenoid composition among natural species of *Mimulus*.

Table 1
HPLC, mass spectrometry and chemical analysis data used to identify *Mimulus* pigments.

HPLC peak no.	Absorption peaks (nm)	No. of epoxide groups	Mass (*m*/*z*)	Identification
1	447, 476	1	584	Antheraxanthin
2	442, 472	2	600	Violaxanthin
3	443, 471	1	584	*cis*-Antheraxanthin
4	437, 466	2	600	*cis*-Violaxanthin
5	448, 477	0	584	Deepoxyneoxanthin
6	443, 472	0	584	*cis*-Deepoxyneoxanthin
7	443, 471 (*trans*), 438, 467 (*cis*)	1	600	Neoxanthin (*cis* or *trans*)
8	443, 473	0	600	Mimulaxanthin

pigment is consistent with that of violaxanthin isolated from spinach (Fig. S1).

Peak 3 appeared only in the nectar guides from *M. lewisii* and *M. kelloggii*, and was observed at 23–24 min (Fig. 3). This pigment was found to have a mass of 584 *m*/*z* and chemical testing revealed one epoxide group, which are also consistent with antheraxanthin [35]. However, the λ_{max} of this pigment is 4 nm blue-shifted from that of the all-*trans* antheraxanthin isomer observed at ~19 min, indicating that this pigment is likely a *cis*-isomer of antheraxanthin and is tentatively identified as such.

Peak 4, observed at 24–26 min (Fig. 3), has a λ_{max} at 437 nm (Fig. 4). Mass spectrometry indicated a mass of 600 *m*/*z* and the chemical test showed evidence of two epoxides which are consistent with the properties of violaxanthin [35]. The 5 nm blue-shift of this peak compared with the spectrum of peak 2, identified as all-*trans* violaxanthin, suggests that the pigment associated with peak 4 is a *cis*-isomer of violaxanthin.

Peak 5 appeared at 29–31 min (Fig. 3). The λ_{max} is at 443 nm (Fig. 4), which indicates that this pigment has a chromophore similar to that of all-*trans* neoxanthin. The chemical test using formic acid yielded no evidence of epoxides. Mass spectrometry indicated the mass to be 584 *m*/*z*. These data suggest that this pigment is deepoxyneoxanthin [35] which has been identified previously as a major component in *Mimulus* flowers [31].

Peak 6, which was only observed in a significant concentration in the nectar guides of *M. lewisii*, shares the same characteristics of deepoxyneoxanthin but has a 5 nm blue-shifted absorption spectrum (Fig. 4). Therefore, this pigment is tentatively identified as a *cis*-isomer of deepoxyneoxanthin.

Peak 7 appears at 32–34 min (Fig. 3). The absorption spectrum of this pigment varied from sample to sample. Interestingly, the extracts from species that only display carotenoids in the nectar guides, which include *M. lewisii*, *M. bigelovii*, *M. torreyi*, and *M. kelloggii*, showed a peak 7 with an absorption spectrum having bands at 438 and 467 nm. The remaining species (e.g. *M. verbenaceus*) that have carotenoids in all the petals showed a peak 7 with absorption bands at 443 and 471 nm. Mass spectrometry and the epoxide test were conducted on representative samples from both phenotypes (*M. lewisii* and *M. verbenaceus*) and the results were found to be identical: The carotenoid had a mass of 600 *m*/*z* and there was evidence of one epoxide group. Furthermore, the retention time of this peak matches that of 9′-*cis* neoxanthin which was observed in the co-chromatography of spinach leaves (Fig. S1, and see [43]). Based on these collective data, peak 7 is identified as neoxanthin. More specifically, based on a comparison of the absorption spectra with published reports [35], peak 7 is identified as all-*trans* neoxanthin in the yellow flowers of *M. guttatus*, *M. cupreus* (yellow form), *M. jungermannioides*, *M. cardinalis*, *M. bicolor*, and *M. verbenaceus*. Peak 7 isolated from the nectar guides of *M. lewisii*, *M. bigelovii*, *M. torreyi*, and *M. kelloggii* displays an absorption spectrum consistent with that of 9′-*cis*-neoxanthin [35]. It should be noted that some of the all-*trans* isomer also appeared to be present in the nectar guide of *M. lewisii*, and some of the *cis*-isomer appeared to be present in the flowers of *M. verbenaceus*, as observed by close examination of the absorption spectra recorded by the PDA detector.

Peak 8, observed at 44–46 min (Fig. 3), shows a λ_{max} at 443 nm (Fig. 4). The pigment showed no evidence of epoxide groups, and was found to have a mass of 600 *m*/*z*. These data, along with co-chromatography with the synthetic mimulaxanthin standard, positively confirm the identity of this pigment as mimulaxanthin, as was previously reported by Nitsche [32].

The expression levels of the carotenoid biosynthetic genes in the *M. lewisii* flower are consistent with its carotenoid composition. The specificity of lycopene cyclase defines a branching point of the carotenoid biosynthesis pathway in plants [28]: lycopene ε-cyclase (LCYE) leads to the production of α-carotene and derived β,ε-xanthophylls (e.g., lutein), whereas lycopene β-cyclase (LCYB) leads to the production of β-carotene and derived β,β-xanthophylls (e.g., violaxanthin, neoxanthin, Fig. 2). In the *M. lewisii* flower, the *LCYE* gene is expressed at negligible level compared with *LCYB1* (>10-fold difference, Table 2), resulting in the flow of all substrates down to the β-carotene branch (Fig. 2) and the complete absence of products on the α-carotene branch (e.g., α-carotene and lutein). The relatively high expression level of the downstream genes along the β-carotene branch (e.g., *MlBCH1*, *MlZEP1*, *MlNSY1*, Table 2) explains the observation that most of the "early products" (i.e., β-carotene, β-cryptoxanthin and zeaxanthin) have been metabolized to the "late products" (i.e., antheraxanthin, violaxanthin, neoxanthin and the neoxanthin-derived deepoxyneoxanthin and mimulaxanthin, Fig. 2) in the *M. lewisii* flower.

Table 2
Gene expression profile from the *Mimulus lewisii* flower transcriptome. The expression level is estimated by the RPKM value. Genes highlighted in shade are expressed at negligible levels.

Gene	Expression level
M1PSY1	312.52
M1PSY2	0
M1PDS1	229.51
M1PDS2	288.57
M1Z-ISO	79.52
M1ZDS1	102.34
M1ZDS2	208.56
M1CRTISO	65.23
M1LCYB1	175.29
M1LCYB2	16.87
M1LCYE	16.48
M1BCH1	236.52
M1BCH2	14.57
M1ZEP1	226.13
M1ZEP2	53.81
M1NSY1	283.94
M1NSY2	14.70

Discussion

This study reports the floral carotenoid composition of the emerging genetic model system *M. lewisii* for the first time, as well as 10 other species that represent four of the six major evolutionary lineages of *Mimulus* (Fig. 1). These data provide a necessary foundation for further genetic analysis on the transcriptional regulation of carotenoid pigmentation using chemically induced *M. lewisii* mutants and natural variation between closely related *Mimulus* species.

The eight peaks revealed by the HPLC analysis of *M. lewisii* flowers are tentatively identified as belonging to just five carotenoid species, antheraxanthin, violaxanthin, neoxanthin, deepoxyneoxanthin and mimulaxanthin: some peaks represent the *trans*- and *cis*-isomers of the same carotenoid species (e.g., peak 1 and 3 are *cis*- and *trans*-isomers of antheraxanthin, Table 1).

Particularly noteworthy among the *M. lewisii* carotenoids are deepoxyneoxanthin and mimulaxanthin, both unusual carotenoids that were originally discovered in *Mimulus* [31,32,34]. Outside of the genus *Mimulus*, deepoxyneoxanthin has been reported to occur in the flowers of *Helianthus annuus*, *T. officinale* and *Impatiens nolitangere* [33], and mimulaxanthin has only thus far been found in *Lamium montanum* [46]. Both pigments are presumably derived from neoxanthin, but the enzymes required for these conversions *in vivo* remain unclear.

The neoxanthin synthase enzyme (NSY) catalyzes the formation of an allene group in place of the C(5,6) epoxide group of violaxanthin to form neoxanthin [47,48]. This enzyme is also a potential candidate for the production of mimulaxanthin, which would simply require a second application of this reaction on the opposite side of the neoxanthin molecule (Fig. 2). This hypothesis can be tested by an enzyme assay using recombinant *M. lewisii* NSY proteins and violaxanthin or neoxanthin as substrates.

It is also possible that NSY could also catalyze the direct formation of deepoxyneoxanthin from antheraxanthin. An NSY-mediated antheraxanthin-to-deepoxyneoxanthin transition would be in substrate-competition with the ZEP-mediated antheraxanthin-to-violaxanthin conversion. In the 11 *Mimulus* species examined, deepoxyneoxanthin only occurs when there is some antheraxanthin present. This may suggest that the less efficient conversion of antheraxanthin to zeaxanthin in these species provides sufficient antheraxanthin to be available as a substrate for conversion to deepoxyneoxanthin. On the other hand, the conversion from neoxanthin to deepoxyneoxanthin may involve a new enzyme yet to be discovered (e.g., a "neoxanthin de-epoxidase"). This mechanism seems highly plausible given the existence of the well-characterized violaxanthin de-epoxidase enzyme [28,49] which is vital to the xanthophyll cycle in the photosynthetic apparatus of green leaves. Neoxanthin has been reported to form deepoxyneoxanthin readily, along with C(5)-hydroxylated deepoxyneoxanthin, upon treatment with the reducing agent lithium aluminum hydride ($LiAlH_4$) [50]. Subsequent treatment of C(5)-hydroxylated xanthophylls with $LiAlH_4$ did not yield C(5,6)-olefinic products confirming that C(5)-hydroxylated deepoxyneoxanthin is not an intermediate in the formation of deepoxyneoxanthin [50]. Based on the lack of C(5)-hydroxylated products in *Mimulus* flowers, it is possible that xanthophyll de-epoxidation *in vivo* also proceeds without a hydroxylated intermediate. Identifying a gene encoding for a neoxanthin de-epoxidase enzyme will require screening for *M. lewisii* mutants that lack deepoxyneoxanthin in their flowers.

Comparison of the carotenoid profiles across species is also revealing. Generally, the more closely related species have more similar carotenoid compositions (Fig. 5). Deepoxyneoxanthin seems to be restricted to the *M. lewisii* and *M. guttatus* clades (Fig. 5), which suggests that deepoxyneoxanthin production evolved in the common ancestor of these two clades. The distribution of mimulaxanthin is more peculiar. It is present in all species belonging to the *M. lewisii* and *M. guttatus* clades and one additional species in the *M. kelloggii–M. torreyi* clade. This distribution can be explained by two alternative scenarios: (1) Mimulaxanthin production evolved only once in the common ancestor of all *Mimulus* species, but was subsequently lost in the *M. whitneyi–M. bigelovii* clade and some species in the *M. kelloggii–M. torreyi* clade; (2) it evolved twice independently in the genus *Mimulus*. Once in the common ancestor of the *M. lewisii* and *M. guttatus* clades, and once in *M. torreyi*. Distinguishing these two possibilities will require denser sampling of species in all major lineages of *Mimulus* in future studies.

Conclusions

This study reports the carotenoid composition from 11 species of *Mimulus*, which were found to contain a total of five carotenoids, including two allenic carotenoids, mimulaxanthin and deepoxyneoxanthin, that are relatively rare in plants. The enzymatic reactions that mediate the biosynthesis of these pigments remain undescribed. The ability to produce deepoxyneoxanthin appears to have originated in a common ancestor of the clades that include *M. lewisii* and *M. guttatus*. The evolutionary origin of mimulaxanthin production is less clear. Furthermore, characterization of the proposed enzymes (e.g. neoxanthin de-epoxidase and the NSY analog) remains to be explored, potentially through enzymatic assay of recombinant proteins in the model species, *M. lewisii*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.abb.2015.03.006.

References

[1] P.M. Beardsley, S.E. Schoenig, J.B. Whittall, R.G. Olmstead, Am. J. Bot. 91 (2004) 474–489.
[2] W. Hiesey, M.A. Nobs, O. Björkman, Carnegie Institute of Washington publ. no. 628 (Washington, DC) (1971).
[3] M.R. Macnair, V.E. Macnair, B.E. Martin, New Phytol. 112 (1989) 269–279.
[4] J. Ramsey, H. Bradshaw, D.W. Schemske, Evolution 57 (2003) 1520–1534.
[5] A.L. Angert, D.W. Schemske, Evolution 59 (2005) 1671–1684.
[6] D.B. Lowry, J.H. Willis, PLoS Biol. 8 (2010) e1000500.
[7] H.D. Bradshaw, S.M. Wilbert, K.G. Otto, D.W. Schemske, Nature 376 (1995) 762–765.
[8] A.L. Sweigart, L. Fishman, J.H. Willis, Genetics 172 (2006) 2465–2479.
[9] M.A. Streisfeld, W.N. Young, J.M. Sobel, PLoS Genet. 9 (2013) e1003385.
[10] Y.-W. Yuan, J.M. Sagawa, R.C. Young, B.J. Christensen, H.D. Bradshaw, Genetics 194 (2013) 255–263.
[11] P.K. Vickery, West. N. Am. Naturalist 52 (1992) 145–148.
[12] D.W. Schemske, H. Bradshaw, Proc. Natl. Acad. Sci. 96 (1999) 11910–11915.
[13] H.D. Bradshaw, D.W. Schemske, Nature 426 (2003) 176–178.
[14] C.R. Owen, H.D. Bradshaw, Arthropod–Plant Interact. 5 (2011) 235–244.
[15] L. Fishman, A. Saunders, Science 322 (2008) 1599–1602.
[16] U. Hellsten, K.M. Wright, J. Jenkins, S. Shu, Y. Yuan, S.R. Wessler, J. Schmutz, J.H. Willis, D.S. Rokhsar, Proc. Natl. Acad. Sci. 110 (2013) 19478–19482.
[17] Y. Brandvain, A.M. Kenney, L. Flagel, G. Coop, A.L. Sweigart, PLoS Genet. 10 (2014) e1004410.
[18] A.M. Cooley, J.H. Willis, New Phytol. 183 (2009) 729–739.
[19] Y. Yuan, J.M. Sagawa, L. Frost, J. Vela, H.D. Bradshaw, New Phytol. 204 (2014) 1013–1027.
[20] Y. Yuan, J.M. Sagawa, V.S. Stilio, H.D. Bradshaw, Genetics 194 (2013) 523–528.
[21] B.J. Glover, Understanding Flowers and Flowering: An Integrated Approach, Oxford University Press, Oxford, 2007.
[22] B. Demmig-Adams, A.M. Gilmore, W.W. Adams III, FASEB J. 10 (1996) 403–412.
[23] B.J. Pogson, K.K. Niyogi, O. Björkman, D. DellaPenna, Proc. Natl. Acad. Sci. 95 (1998) 13324–13329.
[24] K.K. Niyogi, Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 (1999) 333–359.
[25] I. Baroli, K.K. Niyogi, Phil. Trans. R. Soc. Lond. B 355 (2000) 1385–1394.
[26] L. Dall'Osto, A. Fiore, S. Cazzaniga, G. Giuliano, R. Bassi, J. Biol. Chem. 282 (2007) 35056–35068.

[27] P.D. Fraser, P.M. Bramley, Prog. Lipid Res. 43 (2004) 228–265.
[28] M.Á. Ruiz-Sola, M. Rodríguez-Concepción, The Arabidopsis Book/Am. Soc. Plant Biol. 10 (2012).
[29] L. Zechmeister, W.A. Schroeder, Arch. Biochem. 1 (1942) 231–238.
[30] T.W. Goodwin, D.M. Thomas, Phytochemistry 3 (1964) 47–50.
[31] H. Nitsche, Phytochemistry 11 (1972) 401–404.
[32] H. Nitsche, Z. Naturforsch. C 28 (1973) 481–487.
[33] H. Nitsche, C. Pleugel, Phytochemistry 11 (1972) 3383–3385.
[34] H. Nitsche, K. Egger, A.G. Dabbagh, Tetrahedron Lett. 10 (1969) 2999–3002.
[35] G. Britton, S. Liaaen-Jensen, H. Pfander, Carotenoids Handbook, Birkhäuser Verlag, Basel-Boston-Berlin, 2004.
[36] T. Kajikawa, N. Iguchi, S. Katsumura, Org. Biomol. Chem. 7 (2009) 4586–4589.
[37] K. Schiedt, S. Liaaen-Jensen, Carotenoids, Vol. 1A: Isolation and Analysis, Birkhäuser Verlag, Basel-Boston-Berlin, 1995.
[38] T.W. Goodwin, The Biochemistry of Carotenoids, Chapman and Hall, New York, 1982.
[39] A.J. Young, in: A.J. Young, G. Britton (Eds.), Carotenoids in Photosynthesis, Chapman and Hall, London, 1993, pp. 16–71.
[40] G. Britton, S. Liaaen-Jensen, H. Pfander (Eds.), Carotenoids, Vol. 1A: Isolation and Analysis, Birkhäuser Verlag, Basel-Boston-Berlin, 1995.
[41] B.C.L. Weedon, G.P. Moss, in: G. Britton, S. Liaaen-Jensen, H. Pfander (Eds.), Carotenoids, Vol. 1A: Isolation and Analysis, Birkhauser, Basel, Switzerland, 1995.
[42] A. Mortazavi, B.A. Williams, K. McCue, L. Schaeffer, B. Wold, Nat. Methods 5 (2008) 621–628.
[43] S. Takaichi, M. Mimuro, Plant Cell Physiol. 39 (1998) 968–977.
[44] U.D. Neue, HPLC Troubleshooting Guide, A.L.a.W. Corporation, 2002.
[45] T.W. Goodwin, The Biochemistry of the Carotenoids, Vol. I, Plants, Chapman & Hall, London, 1980.
[46] R. Buchecker, C.H. Eugster, Helv. Chim. Acta 63 (1980) 2531–2537.
[47] A. Al-Babili, P. Hugueney, M. Schledz, R. Welsch, H. Frohnmeyer, O. Laule, P. Beyer, FEBS Lett. 485 (2000) 168–172.
[48] A.R. Moise, A. Al-Babili, E.T. Wurtzel, Chem. Rev. 114 (2014) 164–193.
[49] H.Y. Yamamoto, R.M. Higashi, Arch. Biochem. Biophys. 190 (1978) 514–522.
[50] B.P. Schimmer, N.I. Krinsky, Biochemistry 5 (1966) 3649–3657.
[51] D.L. Grossenbacher, J.B. Whittall, Evolution 65 (2011) 2712–2718.