Which pigment appears first in the corolla—patterned or background?

Eapsa Berry (eapsa321@gmail.com)
University of Delhi https://orcid.org/0000-0001-7089-7157
R. Geeta
University of Delhi https://orcid.org/0000-0002-8498-470X

Short Report

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SHORT COMMUNICATION

Which pigment appears first in the corolla—patterned or background?

Eapsa Berry[1] and R. Geeta[1*]

[1]Department of Botany, University of Delhi, Delhi 110007, India

[*]Corresponding author

E-mail: rgeeta53@gmail.com

MAIN CONCLUSION

We show that background and patterned pigmentation on corolla can appear either simultaneously or successively with the predominance of the case of pigment pattern developing earlier than background pigmentation.

ABSTRACT

Flowers display a diversity of pigment patterns on petals – spots, stripes, blotches, and varying combinations of these. Such pigment patterns are accompanied and surrounded by a background that is a contrasting shade or colour sometimes white. We ask the question: Do the pattern and background colours appear simultaneously or successively, and if the latter, is there a bias in which one appears first? We studied the morphological development of flowers of 35 species containing both types of pigmentation, sampled from clades across angiosperms (monocots, Ranunculales, Caryophyllales, rosids and asterids) to address this question of timing of occurrence of the two types of pigmentation. In 28 of the species studied, pigment pattern started appearing in the corolla earlier than the background colour. Pigment pattern appeared later in four cases, and simultaneously with background colour in three cases. Thus, our results reveal, for the first time, variation in developmental sequence of pattern and background colour, with an apparent tendency toward earlier appearance of pigment pattern in the corolla. We hypothesize that the mechanisms involve the imperatives of pigment types, reaction kinetics, differential gene expression, and reaction-diffusion models.

KEY WORDS

background pigment, corolla, development, morphogenesis, pigment pattern, timing

INTRODUCTION

In angiosperms, flowers, more than any other organ, display a large variety of pigment patterns. Floral colour patterns are localized in clearly confined and generally well-defined areas on the corolla to form dots, streaks, and blotches (Wheldale-Onslow 1925; **Fig. 1**). Such pigment patterns are present on, or are surrounded by, a background of either contrasting colour or contrasting shade of a similar colour or on a non-pigmented background. Pigment patterns usually, though not always, are more intensely coloured than the background. In some flowers, pigment patterns develop in distinct regions of the corolla (e.g., petal lobe, throat region, veins), while in others, the area of pigment pattern is not well-defined, resulting in blurred boundaries (Drews et al. 1992). Pigments occur mostly in the adaxial epidermal cells of petals, but sometimes in both abaxial and adaxial epidermal cells (Kay et al. 1981) Pigment synthesis is initiated at very early stages of the bud, as observed in *Antirrhinum majus* L. (Plantaginaceae)*, Petunia hybrida* E.Vilm. (Solanaceae) and *Gorteria diffusa* Thunb. (Asteraceae) (Coen et al. 1986; Martin and Gerats, 1993; Thomas et al. 2009). Petal development can be broadly divided into two phases: cell division (slow growth) and cell expansion (fast growth) with duration of the phases varying from species to species (Martin and Gerats, 1993; Weng et al. 2011; Landis et al. 2016) Pigment formation starts when the petal enters the second phase, but regulatory gene expression is maximum in the final stages of the first phase (Coen et al. 1986; Martin and Gerats, 1993)

A pigment pattern on the corolla, both in its presence and localization or design, is generally a stable, heritable trait that appears consistently in succeeding generations, with its appearance governed by genes, its shape being either regular (geometric) or irregular.

Pigment patterns other than stable, heritable patterns also exist – e.g., colour break patterns (Hunter et al. 2011) but are not the subject of this study. There are various types of pigment patterns on corolla (Fig. 1), and the major types are streaks, spots, and blotch/es (Wheldale-Onslow, 1925; pers. obs.).

It is known that the expression of pigments (anthocyanins, carotenoids, betalains, chlorophylls) are regulated by R2R3-MYB transcription factors (TF) (Stracke et al. 2001, Hatlestad et al. 2015; Sagawa et al. 2016; Ampomah-Dwamena et al. 2019) In the model plant, *Arabidopsis thaliana* (L.) Heynh., anthocyanin pigmentation is regulated by subgroup 6 members of the R2R3-MYB TF family by interacting with bHLH TF and WD repeat proteins (Walker et al. 1999; Stracke et al. 2001; Matsui et al. 2004; Gonzalez et al. 2008; Dubos et al. 2010) The regulatory complex of R2R3-MYB, bHLH, and WD40 interact to bring about anthocyanin pigmentation and patterning in flowers, fruits, seeds, and leaves in *Zea mays* L. (Poaceae), *Antirrhinum majus* L., *Petunia hybrid* Juss., *Mimulus gattatus* L. (Phrymaceae), and *Ipomoea nil* L. (Convolvulceae) (Ramsay & Glover, 2005, Davies et al. 2012). Various studies suggest that R2R3-MYB genes are the primary activators of pigments biosynthesis in petals (Elomaa et al. 2003; Morita et al. 2006; Schwinn et al. 2006; Nakatsuka et al. 2008; Chiou and Yeh, 2008; Ma et al. 2009; Shang et al. 2010; Yamagishi et al. 2010; Albert et al. 2011; Ohno et al. 2011; Yuan et al. 2014; Sagawa et al. 2016) and that R3-MYB genes act as repressors (Ding et al 2020, Zang et al. 2020) The morphogenesis of colour patterns is based on the regulated spatio-temporal expression of R2R3-MYB, bHLH, and WD40 TF genes (Schwinn et al. 2006). Post-transcriptional regulation by small RNAs has also been indicated in pigment biosynthesis in flowers (Davies et al. 2012). Apart from MYB and bHLH regulators,

78 RNA interference and microRNA (miRNA) based regulation also affect pigment patterns
79 (Matsubara et al. 2012).

80 The purpose of this study was to understand one aspect of the development of corolla
81 pigment patterns (CPP) in angiosperms. The main question addressed in this study: Is the
82 emergence of the two types of pigmentation on the developing petal (background and
83 patterned) simultaneous or successive?

84 MATERIALS AND METHODS

85 *Morphological developmental study*
86 A survey of morphological development of CPP and background pigmentation was done on
87 35 plant taxa sampled across angiosperm (Fig. 2). Care was taken to have as broad sampling
88 as possible across the angiosperms, given limitations of availability. We have included
89 monocots, Ranunculales, and core-eudicots -- Malvidae (rosid II) and Fabidae (rosid I),
90 Caryophyllales, and seven families from asterids – asterid I (six families) and asterid II (one).
91 All were cultivated species, most of which were growing in the Botanical Garden of the
92 Department of Botany, University of Delhi, Delhi, India, and some in the university
93 compound of Universidad Nacional Autónoma de México, México City, Mexico. The
94 morphological development, from genesis to maturity, of pigment pattern and background
95 pigment was observed, recorded, and analyzed.

96 Floral buds of different sizes and developmental stages based on pigment development
97 were harvested from 3-4 plants of each species. The buds for each species were arranged
98 in order of increasing size and/or advancing stage. The corollas of flowers at different

developmental stages were split open and placed on a contrasting background, and their images captured. Small buds < 5 mm in length were photographed under a stereo microscope (Zeiss Stemi 305). The younger the bud, the more difficult it was to flatten the corolla and the use of a drop of tap water and paint brush (size '0') allowed easy flattening and smoothening of petals on glass slides. Sometimes glass plates were placed on the petals to hold them down; and sometimes additional plasticine clay support was given to flatten the curled petals. Most images were captured by Nikon D200 or D5100.

To illustrate the phylogenetic distribution of the sampled species, a phylogenetic tree based on APG IV system of classification (Stevens P. F. 2001 onwards) was drawn in Mesquite 3.5 (Maddison and Maddison, 2015) and edited in FigTree v. 1.4 (Rambaut, 2009).

Estimation of the relative percentage of flowers with both background and patterned pigmentation

In order to estimate the frequency of flowers with background and patterned pigmentation across angiosperms, we investigated a sample of 525 flowering plant species, from the dataset of Soltis et al. 2011, in which the sampling is assumed to have been random with reference to pigmentation type. We scored the presence and absence of background pigmentation and/or pigment patterns by observing photographs of flowers from online databases. Using the scored data, the proportion and percentage of flowers with both background and patterned pigmentation was estimated. While white colour of plant tissue is said to be due to the total reflection of light in the absence of pigment (Peach, 1955), these tissues may include UV-reflecting or absorbing areas visible to pollinators, but we did not

include flowers with CPP on human-white background in our study as we were interested in pigment development.

RESULTS AND DISCUSSION

The sequence of appearance of CPP and background pigmentation was observed and recorded for 35 species (Fig. 3, Supplementary Table S1). Pigment pattern was found to start developing first, and the background pigment appeared later in corollas of 28 of the 35 species examined. In four species background pigmentation appeared earlier, while in the remaining three species patterned and background pigmentation appeared simultaneously. In general, for both CPP and background, pigmentation first appeared in the central part of the future total pigmented area of the petal, irrespective of whether it was restricted to the proximal, distal, or central region on the petal. Pigment development was divided into two categories with respect to the mode of localization: **on-vein** and **off-vein** ('off-vein' referring to the inter-vein regions). In the on-vein category, the pigment starts developing exclusively on the epidermal regions above the veins and then spreads to include the off-vein epidermal regions of total pigmentation (e.g., *Ruellia simplex* C. Wright). On the other hand, in the off-vein category, pigmentation starts first in the regions in between veins (e.g. *Ruellia tuberosa* L.; Supplementary Fig. S1). In both cases, the pigmentation starts from the central part of the total assignable area gradually developing to include more area in a centrifugal pattern maintaining an apparently uniform rate.

We observed that in all the four cases where the background pigment appeared first (*Canna indica* L., *Eschscholzia californica* Cham., *Caesalpinia pulcherrima* (L.) Sw. and *Tagetes tenuifolia* Cav.), the pigment combination is yellow background with orange/red CPP (Fig. 2**,** Supplementary Table S1).

To assess the prevalence of flowering plant species with both background and pigment pattern, we investigated 525 plant species in the study of Soltis et al. (2011). These species

belong to 62 orders (of a total of 64) and 298 families (of 416) according to the APG IV system of classification (Chase et al. 2016). We found that 66 of the 525 species (less than 13%) showed both background and patterned pigmentation. These 66 plant species belonged to 48 plant families in 22 plant orders, of which more than 50% representation was from the five eudicot plant orders Lamiales (8 families), Malpighiales (7 families), Asterales (3 families), Caryophyllales (3 families), Ericales (3 families) and Ranunculales (3 families) of the total of 62 orders in the study (see Supplementary Table S2 and Fig S2). Adding to this the number of species (41 spp.) with patterned pigmentation on white/colourless background, this makes up a total of 107 of 525 plant species that show patterned pigmentation. Most of the species in the angiosperm study (~80%) had flowers that were non-patterned, very small, or had no perianth. It is noteworthy that some clades appear to be consistently non-patterned (Supplementary Fig. S2). Our results suggest that our own observational study may be a reasonable representation of the development of flower pigmentation across angiosperms.

In our study, we found all possible sequences of development – CPP appearing first, i.e, before background pigmentation started (28 of 35 species); CPP appearing later than background pigmentation (four species); and both types of pigmentation appearing simultaneously (three species). This raises the following questions: (i) are there plausible processes that might underlie the preferred developmental sequence in a large proportion (80%) of flowers observed; and (ii) are there features that distinguish the flowers of species that exhibit other developmental sequences; and (iii) could the sequence of pigment appearance in a species or taxon depend on the type of pigments involved? We consider these questions in the light of plausible underlying genetic, biochemical, and developmental factors that could explain the apparent general pattern and departures from this pattern that we observed.

Early and late gene expression of pigmentation in flower development show temporal difference in expression of pigmentation of two types. In *Clarkia gracilis* A. Nelson & J.F. Macbr. (Onagraceae), different copies of the dihydroflavonol reductase (DFR) gene are expressed at different stages of bud development and are expressed either in the CPP or in the background (Martins et al. 2013).

Intense and more pigmented anthocyanin patterned pigmentation appear earlier than lighter background pigmentation. In petals of Xibie tree peony (*Paeonia* spp., Paeoniaceae) the blotch regions (CPP) contain higher levels of anthocyanin compared to the background region, conferring a darker colour to blotches (Zhang et al. 2007). Deeper blue flowers of *Torneia fournieri* Lind. (Linderniaceae) are produced when anthocyanins make complexes of co-pigments with flavones or flavonols; in some cases the complexes are elaborate and consist of six anthocyanin and six flavone molecules and two metal ions (Goto and Kondo, 1991; Aida et al. 2000). It is likely that in most flowers there exist quantitative and qualitative differences in the sets of pigment molecules respectively in CPP and background regions. As most of the above pigments may be supposed to involve similar pigment biochemical pathway (e.g., flavonoid biosynthetic pathway: Martins et al. 2013), the kinetics of the reactions are likely to involve a rate-limiting step triggering controlled accumulation of pigment biomolecules. For instance, in the flavonoid biosynthetic pathway, substrate competition between enzymes occurs to produce differences in the relative quantities of anthocyanins and flavonols resulting in different pigmentation outcomes (McCarthy *et al.* 2017, 2020). Therefore, one can speculate that, for the accumulation of relatively large quantities of the same pigment molecule (quantitative difference) or a greater variety of pigment molecules (qualitative difference), CPP development might have to start early

enough to allow this accumulation well in time before the flower bud opens. The differential timing of expression would be regulated by TFs such as R2R3-MYB genes (Schwinn et al. 2006; Albert et al. 2011). The temporal and spatial separation of patterned and background pigmentation is governed by reaction-diffusion kinetics. Regulated by the interaction of R2R3-MYB and R3-MYB proteins, that corresponds to an activator-inhibitor system of reaction-diffusion model, spot patterns are formed in *Mimulus* flower petals (Ding et al. 2020).

Developmental timing of anthocyanin, chlorophyll, and carotenoid expression is different. In cases where background pigmentation appears either simultaneously with or earlier than patterned pigmentation, we suggest that this may occur in those instances where a) the background and patterned areas do not show significant differences in concentration of pigment molecules -- this type of pigmentation might not involve enzymatic competition, or b) entirely different classes of pigments occur in the two regions (e.g., anthocyanins and carotenoids). It has been observed that chlorophyll and anthocyanins express early in the time of flower development and carotenoids develop later in the development process (Xue at al., 2019). Carotenoids and anthocyanins have distinct biosynthetic pathways as well as sites of synthesis and occurrence. Carotenoids are known to be synthesized and stored in plastids (chromoplasts in flowers and fruit) (Sun et al. 2018), whereas anthocyanins are synthesized in the cytosol and get stored in vacuoles (Kitamura, 2006). The latter scenario is suggested by our observation that, in the four cases where the background pigment appeared first, there was yellow background with orange/red CPP, presumably involving carotenoids. This might reflect a limitation of our study due to inadequate sampling or, alternatively might prove an interesting pointer demanding further investigation. In the cases where the background pigmentation and the patterned pigmentation appear simultaneously, then there could be the following steps: i) flowers of

different species show variable time duration of development in cell division and cell elongation phases (Martin & Gerats, 1993), therefore, sometimes the time periods of appearance coincide due to short cell elongation phase, ii) in short cell elongation phase mutually exclusive localizations of background and patterned pigmentation comprising of different pigment molecules (with no substrate competition) appear simultaneously, e.g. *Thunbergia erecta* the blotch pigmentation is produced by carotenoids (yellow/orange) and the background is made up of anthocyanins and both appear simultaneously.

Deeper mechanistic insights would help understand the biology of corolla pigmentation and also yield information for horticulturists to experiment with CPP in closely related taxa for generating new hybrids or engineered flowers, e.g. roses with background pigmentation along with stripes and blotches. Comparative plant and animal pigmentation studies could yield better understanding of the biology of cellular and tissue pigmentation in general.

CONCLUSIONS

Not much is known about the modes of development of corolla pigmentation patterns in angiosperms. We show that all three conceivable modes of pigmentation development on corolla do occur, while equally significantly there is a strong case – of CPP developing earlier compared to background pigmentation – in favour of seeking various common underlying mechanisms. The present study is a small step toward better understanding of the evolution and development of CPP in angiosperms.

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AUTHOR CONTRIBUTIONS

EB and RG contributed substantially and equally to each of the following: conception and design of the work, analysis and interpretation of results, and writing the manuscript; EB was responsible for acquiring the data. Each author has given final approval of the version to be sent for publication; and each has agreed to be accountable for all aspects of the work in order to ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

DATA ACCESSIBILITY STATEMENT

No data used in the study has been archived in public accessible repositories; all data related to the study has been fully described in the manuscript; and photographs of species not published are available on request.

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SUPPORTING INFORMATION

Table S1. Description of the observed developmental order of background and pattern pigmentation on corolla in the taxa studied.

Fig. S1 Figure illustrates two vein-related types of development of corolla pigment pattern in two species of *Ruellia*. **a, c, e,** and **g** are images of *R. simplex* where **c** (1.4 cm bud)**, e** (1.5 cm bud)**,** and **g** (3 cm bud) show 'on-vein' blotch pattern development. Again, **b**, **d, f,** and **h** are images of *R. tuberosa* where **f** (1.8 cm bud)**,** and **h** (3 cm bud) show 'off-vein' blotch pattern development, whereas a bud of smaller size **d** (1.4 cm) shows no pigmentation. In *R. simplex* pigment pattern emerges in stage II when the corolla elongates and appears outside the covered apical part of the calyx; in *R. tuberosa* pigmentation appears in stage III when corolla tube bulges and spreads —pigmentation occurs rapidly in the case of *R. tuberosa*

Table S2. Scored data on pigment pattern and background pigmentation for 525 flowering plant species from the dataset of Soltis et al. 2011.

Fig S2 Displaying taxa with pigmentation types on the Soltis et.al. (2011) angiosperm phylogeny. Taxa showing presence of pigment pattern and background (non-white) pigmentation are marked with solid red circles, and taxa with pigment pattern on a white background are shown using red hollow circles

FIGURE LEGENDS

Fig. 1 Illustration of the different forms of corolla pigment pattern:

(**a**-**m**) stable pattern (**n**, **o**) unstable pattern (**a**) background colouration (e.g. $^{\Psi}$*Barleria prionitis*; Acanthaceae) (**b**) spot pigmentation pattern (e.g. $^{\Psi}$*Rhododendron triflorum*; Ericaceae) (**c**, **d**) streak pattern (e.g. $^{\Psi}$*Duranta erecta*; Verbenaceae) (**e**) band pattern ($^{\Psi}$*Andrographis paniculata*; Acanthaceae) (**f**) blotch pattern ($^{\Psi}$*Rhododendron dalhousiae*; Ericaceae) (**g**, **h**) composite pattern ($^{\Psi}$*Dicliptera paniculata* and $^{\Psi}$*Justicia simplex*; Acanthaceae) (**i**) picotee pattern [*Aquilegia vulgaris*; Ranunculaceae (Kristofferson 1922) and *Papaver rhoeas*; Papaveraceae (Newton 1929)] (**j**) colour tinge/flush (**k**) bud-blush pattern (e.g. $^{\Psi}$*Allamanda blanchetii*; Apocynaceae) (**l**) bull's-eye pattern (whole flower) [e.g. *Argentina anserine* (Koski and Ashman, 2014)] (**m**) star pattern (whole flower) (e.g. $^{\Psi}$*Ipomea nil*) (**n**) colour-break pattern leading to formation of bicolour flowers [e.g. in daffodils, tulips and lilies (Hunter et al. 2011)] (**o-i**, **o-ii**) chimeric pattern showing two distinct flowers from the same plant [e.g. *Mirabilis jalapa*; Nyctaginaceae (Demerec, 1935)] $^{\Psi}$ – pers. obs.

Fig. 2 Taxa sampled

449 Out of the 35 species studied 28 showed CPP development starting earlier than the
450 background pigmentation; four taxa, that are marked with pink coloured branches,
451 showed precedence of background pigmentation over CPP; and three taxa here marked
452 with a blue branch had CPP and background pigmentation emerging simultaneously
453

454 **Fig. 3 Stages in the development of CPP and background pigmentation in representative**
455 **species**

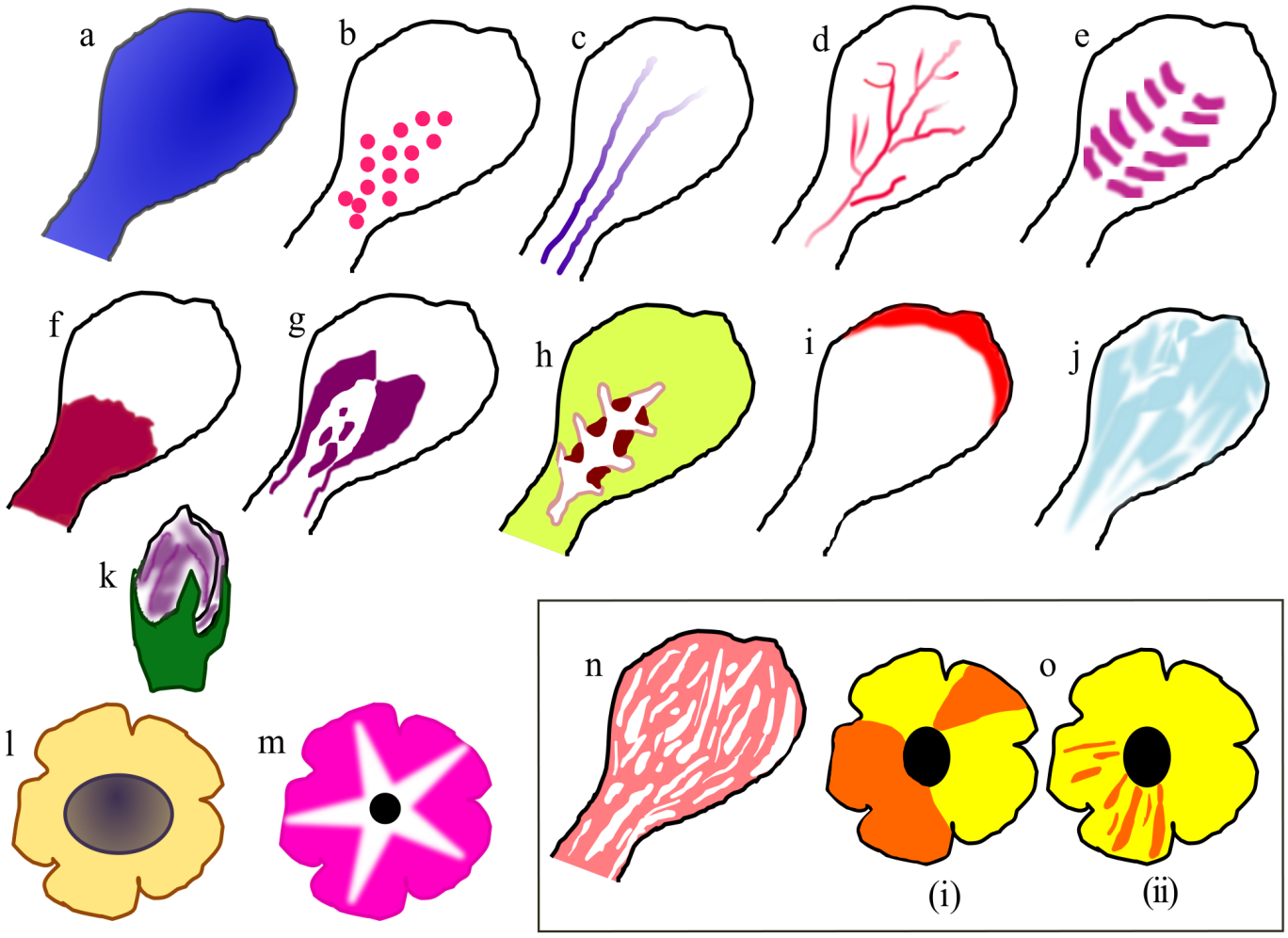
456 **a-f** *Campsis radicans***,** *Digitalis purpurea*, *Catharanthus roseus*, *Dianthus* sp., *Nemesia*
457 sp., and *Eschscholzia californica*. **a-1, b-2, c-2, d-1,** and **e-1** show CPP appearing earlier
458 compared to background pigmentation in *Campsis radicans***,** *Digitalis purpurea*,
459 *Catharanthus roseus*, *Dianthus* sp., and *Nemesia* sp. and **f-1** shows CPP appearing after
460 background pigmentation in *Eschscholzia californica*

461

462 **Figure 4. Model for the early appearance of pattern pigmentation compared to the**
463 **background**
464 Three hypotheses (not mutually exclusive), presented starting from the bottom and
465 connected by black arrows, may in combination suggest the early development of
466 pigment pattern observed in the study. I (bottom), *Reaction-diffusion*: Interactions
467 between morphogens – an activator (e.g., hormone, TF, or miRNA) and an inhibitor – are
468 indicated by straight arrows. Interaction of the activator with the inhibitor molecule
469 inhibits, whereas accumulation of the activator triggers the localized expression of
470 pigmentation. The nature of the 'trigger" (red star) is unknown, but could be the activator
471 molecule itself, and is likely set off during the cell division phase. II (middle),

Differential gene expression: The trigger activates transcription factors R2R3-MYB (sub-group 6) (yellow star, early-acting; orange star, late-acting) that differentially regulate pigment-synthesizing structural genes coding for enzymes of the flavonoid biosynthetic pathway, e.g., dihydroflavonol reductase (DFR); different copies of DFR are activated at different times -- the early-expressing copies act in the CPP region, and the late-expressing copies act in the background. III (top), *Reaction kinetics*: Early formation of pigment molecules in the CPP region allows time for accumulation of greater amount (quantitative) and variety (qualitative) of pigments as might be required by the reaction rate kinetics of the biochemical pathway, possibly including a rate-limiting step (blue star) The dashed circles indicate CPP regions; blue and purple shapes (triangle, rhombus, pentagon and hexagon) represent different pigment molecules in the CPP and background region. Differential expression of DFR genes and their role in pigmentation, and reaction kinetics DFR gene products, is known; a role for reaction-diffusion is plausible but needs to be tested.

Figures



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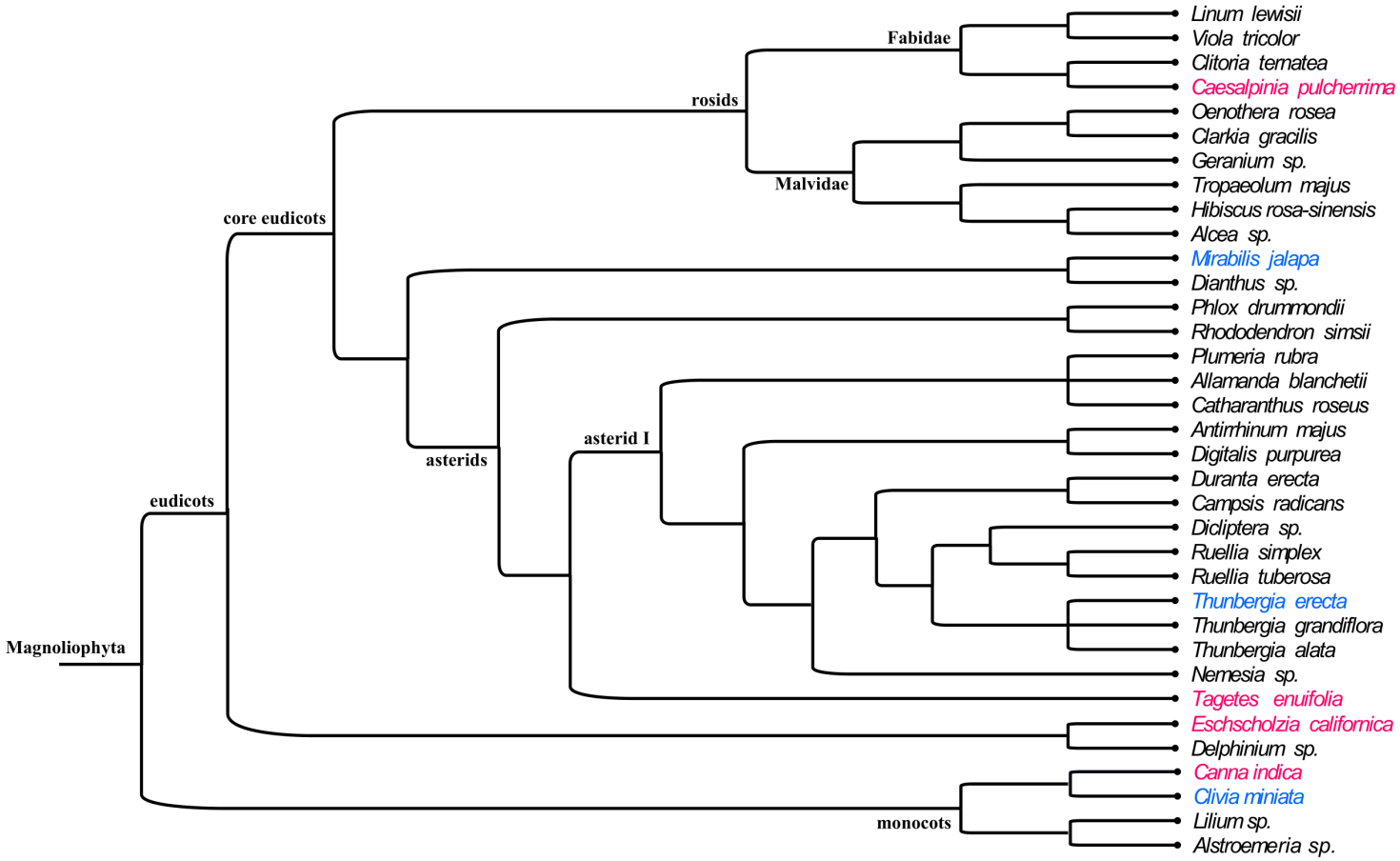

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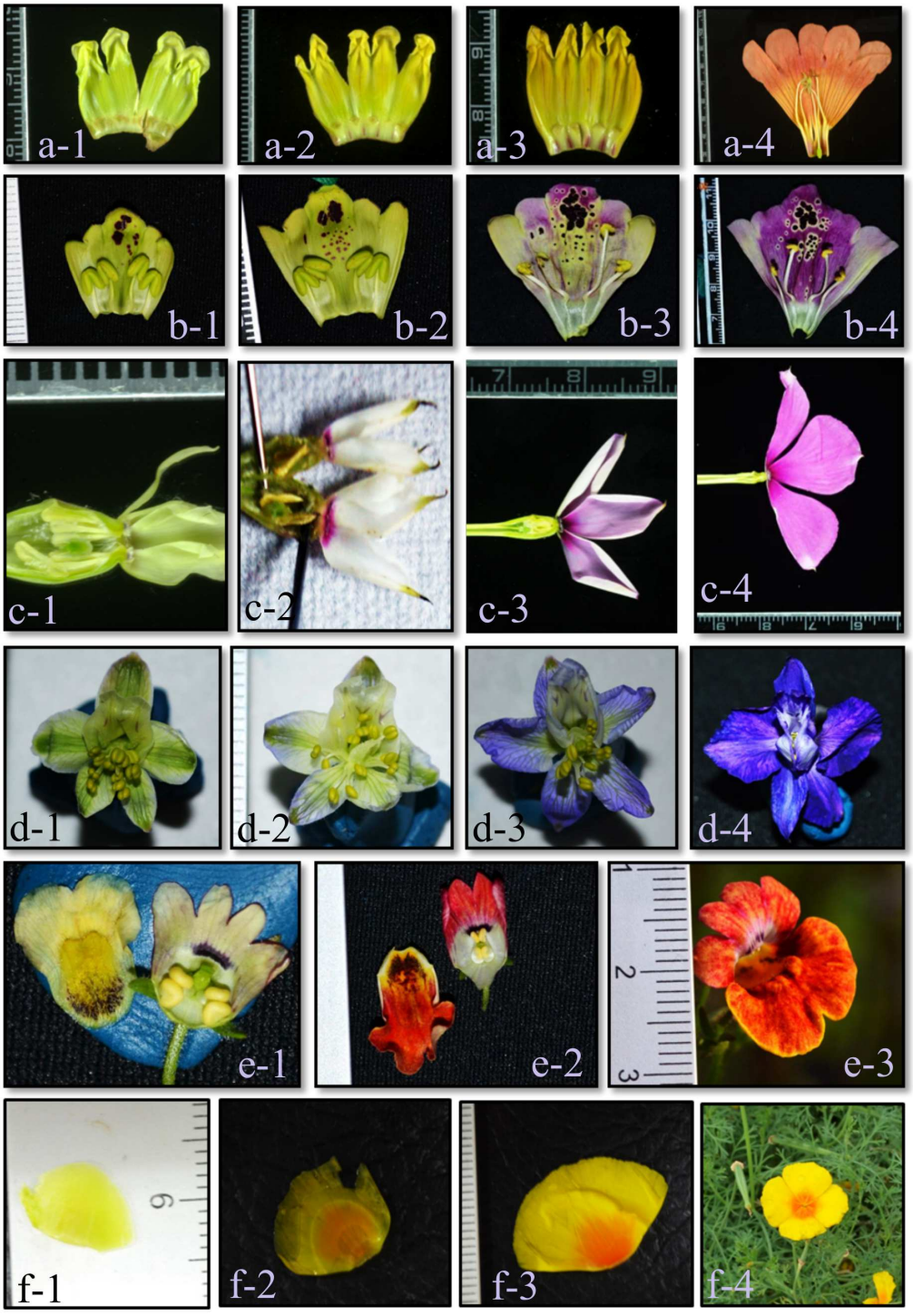

Figure 3

Figure 3. Stages in the development of CPP and background pigmentation in representative species

a-f Campsis radicans**,** Digitalis purpurea, Catharanthus roseus, Dianthus sp., Nemesia sp., and *Eschscholzia californica*. a-1, b-2, c-2, d-1, and e-1 show CPP appearing earlier compared to background

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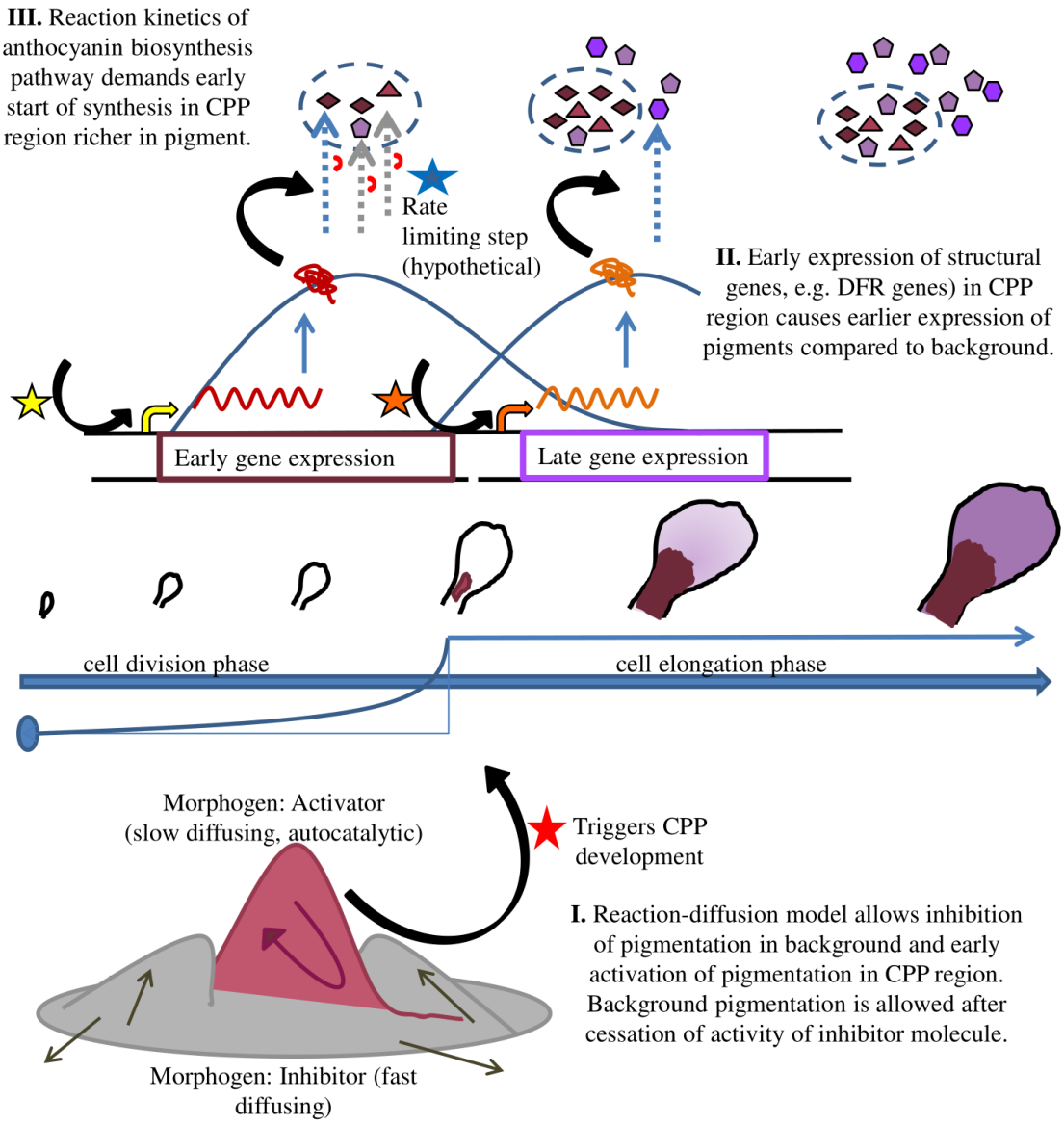

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Supplementary Files

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