Phytochemistry xxx (2016) 1–9

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Evolutionary correlations in flavonoid production across flowers and leaves in the Iochrominae (Solanaceae)

Andrea E. Berardi [a, *], Sherry B. Hildreth [b], Richard F. Helm [c], Brenda S.J. Winkel [b], Stacey D. Smith [a]

[a] Department of Ecology and Evolutionary Biology, University of Colorado-Boulder, Campus Box 334, Boulder, CO, 80309, USA
[b] Department of Biological Sciences, Virginia Tech, Blacksburg, VA, 24061, USA
[c] Department of Biochemistry, Virginia Tech, Blacksburg, VA, 24061, USA

ARTICLE INFO

Article history:
Received 13 January 2016
Received in revised form
12 May 2016
Accepted 23 May 2016
Available online xxx

Keywords:
Iochroma
Solanaceae
UPLC
HPLC
Mass spectrometry
Flavonoids
Flavonols
Anthocyanins
Correlated evolution

ABSTRACT

Plant reproductive and vegetative tissues often use the same biochemical pathways to produce specialized metabolites. In such cases, selection acting on the synthesis of specific products in a particular tissue could result in correlated changes in other products of the pathway, both in the same tissue and in other tissues. This study examined how changes in floral anthocyanin pigmentation affect the production of other compounds of the flavonoid pathway in flowers and in leaves. Focusing on the Iochrominae, a clade of Solanaceae with a wide range of flower colors, liquid chromatography coupled with mass spectrometry and UV detection was used to profile and quantify the variation in two classes of flavonoids, anthocyanins and flavonols. Purple, red, orange and white-flowered Iochrominae produced all of the six common anthocyanidin types, as well as several classes of flavonols. Differences in anthocyanin and flavonol production were significantly correlated in flowers, particularly with respect to B ring hydroxylation pattern. However, these differences in floral flavonoids were not strongly related to differences in leaf chemistry. Specifically, most species made only flavonols (not anthocyanins) in leaves, and these comprised the two most common flavonols, quercetin and kaempferol, regardless of the color of the flower. These results suggest that shifts in flower color may occur without significant pleiotropic consequences for flavonoid production in vegetative tissues. Similar studies in other systems will be important for testing the generality of this pattern in other groups of flowering plants.

1. Introduction

Plants contain a relatively small number of genes that code for enzymes in biochemical pathways, which collectively produce thousands of different specialized metabolites (Pichersky and Gang, 2000). The organization of genes into metabolic and biochemical pathways varies, but a unique and emergent feature of many of these systems is that precursor substrates, enzymes, and regulatory elements are shared by several different groups of metabolites (Jeong et al., 2000; Ravasz et al., 2002; Schilling and Palsson, 1998). This connectivity of substrates and enzymes creates the potential for correlated changes at the molecular level (Xi et al., 2011), as well as in the resulting biochemical phenotypes and traits (Armbruster, 2002). Correlated trait variation has important consequences for both wild and domesticated species, as it can limit the response to directional selection or result in undesirable phenotypes (Chen and Lübberstedt, 2010; Otto, 2004).

The ubiquitous plant flavonoid pathway produces a diverse array of compounds from a small set of substrates, enzymes, and regulatory elements, providing a promising system for studying correlated biochemical variation within and across tissues. Most plant tissues accumulate multiple types of flavonoids, which may serve as pigments, antioxidants, defense compounds, and signaling molecules (Chalker-Scott, 1999; Winkel-Shirley, 1996). For example, many flowers produce anthocyanins (red, blue and purple flavonoid pigments) as well as flavonols, a group of flavonoids that act as co-pigments and create UV-patterning visible to insects (Gronquist et al., 2001; Koski et al., 2014). Moreover, a single type of flavonoid is often found in multiple tissues. For example, while anthocyanins are abundant in colored flowers and fruits, they can also be produced in vegetative tissues in response to light and

* Corresponding author.
E-mail address: berardi.andrea.e@gmail.com (A.E. Berardi).

http://dx.doi.org/10.1016/j.phytochem.2016.05.007
0031-9422/

temperature stress (Lee and Gould, 2002).

Building on the well-known flavonoid pathway (Fig. 1), it is possible to make predictions about correlated variation among its products. First, because different classes of flavonoids share several enzymatic steps, changes in the expression and/or activity of these enzymes could generate correlated shifts in multiple pathway products. For example, elevated expression of any of the upstream enzymes (CHS, CHI) could increase overall flux through the pathway and lead to increases in the multiple downstream products. Second, more fine scale correlations might be expected among different classes of flavonoids, such as in the hydroxylation patterns of the B ring (Halbwirth, 2010). For example, an increase in the activity of F3′5′H should result in greater production of the trihydroxylated flavonol, myricetin (**4**), as well as the trihydroxylated anthocyanidin, delphinidin (**8**, Fig. 1). If such a shift toward trihydroxylated flavonoids depleted the substrates available for mono- and di-hydroxylated forms, negative correlations would be expected in production between these forms.

To the degree that the pathway and its regulation are shared across the plant, correlations in biochemical variation may also extend across multiple tissues. Species that produce particular classes of anthocyanins or flavonols in flowers often produce those same compounds in leaves and other vegetative tissues (Berardi, 2014; Goss, 1973; Harborne, 1977; Lawrence et al., 1939; Lowry et al., 2012; Martin et al., 1991). However, flowers and leaves experience different selective pressures and perform distinct ecological roles; thus they also produce metabolites specialized for their functions (Harborne, 1993; Strauss and Whittall, 2006; Wessinger and Rausher, 2012). The extent of co-variation in flower and leaf flavonoids will depend on the specialization of transcriptional factors and other regulatory elements (Quattrocchio et al., 1999; Stracke et al., 2007; Streisfeld and Rausher, 2009) and of the enzymes themselves, when they exist in multiple copies (Koes et al., 1989).

This study focuses on the tomato family, Solanaceae, in which flavonoid genes typically occur in single copies (e.g. *FLS*, *F3H*, *DFR*, De Jong et al. (2004)). Even when multiple copies are present, one of the genes is predominantly expressed across the plant (e.g., *CHS*: Koes et al. (1989) and *CHI*: van Tunen et al. (1988)). These structural genes are regulated by a complex network of transcription factors, some of which are tissue specific (Ramsay and Glover, 2005) but others of which act throughout the plant (Albert et al., 2014). The sharing of transcriptional regulators and expression of specific genes across tissues would predict correlated biochemical composition. Such patterns have been qualitatively assessed in a handful of Solanaceae species (Lewis et al., 1998; Taylor, 1940), but not tested using statistical or comparative methods.

This body of knowledge is expanded here by quantifying flavonoid composition in flowers and leaves across the Iochrominae, an ornamental clade of Solanaceae known for its wide range of flower colors (Smith and Baum, 2006). Previous studies have shown that this color diversity is likely driven by competition for pollinators (Muchhala et al., 2014), and in some species is associated with changes in floral anthocyanin production (Smith and Rausher, 2011). These changes in anthocyanin production could lead to correlated changes in other floral flavonoids as well as changes in the chemical composition of non-floral tissues.

Focusing on a set of species representing the different color phenotypes across the clade, a combination of biochemical profiling and statistical phylogenetic analyses were used to track the changes in flavonoid production through flower color transitions. Specifically, the following questions were addressed: (i) what

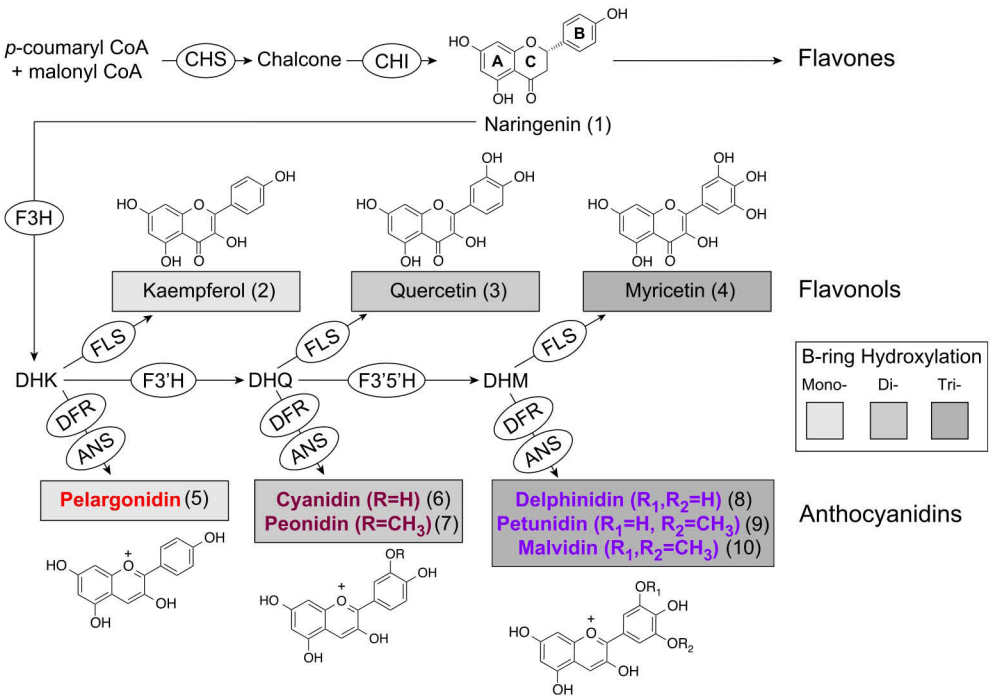

Fig. 1. The flavonoid pathway adapted from Rausher (2008). Pathway enzymes include: CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3-hydroxylase; FLS, flavonol synthase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; and UFGT, UDP flavonoid glucosyl-transferase. Precursor compound abbreviations are DHK, dihydrokaempferol; DHQ, dihydroquercetin; DHM, dihydromyricetin. Products of the pathway include flavonols (kaempferol, quercetin, and myricetin, **2**–**4**) and anthocyanidins (pelargonidin, **5**; cyanidin and its methylated derivative peonidin, **6**–**7**; and delphinidin and its methylated derivatives petunidin and malvidin, **8**–**10**). These anthocyanidins can be converted into multiple glycosylated and acylated anthocyanins (not shown). Other types of flavonoids, such as flavanones and condensed tannins, are not shown as they are rare in the Solanaceae (Eich, 2008). Compound numbers that correspond to those in the text are provided next to the compound name.

Please cite this article in press as: Berardi, A.E., et al., Evolutionary correlations in flavonoid production across flowers and leaves in the Iochrominae (Solanaceae), Phytochemistry (2016), http://dx.doi.org/10.1016/j.phytochem.2016.05.007

is the range of flavonoid diversity in flowers and leaves of Iochrominae?; (ii) do shifts in floral anthocyanins lead to correlated changes in other floral flavonoids?; and (iii) do these correlated changes extend to leaf flavonoid composition? This is only the second study to apply comparative methods to testing for biochemical correlations (Armbruster, 2002), and the first to apply these methods to quantitative (as opposed to qualitative) biochemical data. Understanding the extent of correlated variation in floral pigmentation and flavonoid production across the plant in this diverse clade will provide new insight into the range of pleiotropic consequences that may result from natural or artificial selection acting on flower color.

2. Results

2.1. UPLC-MS metabolite profiling and compound identification

Metabolite profiling was performed in order to characterize the flavonoid composition of eight species, representing white, purple, red, and orange floral colors (Supplementary Table 1), using ultra performance liquid chromatography coupled with electrospray ionization and quadrupole time-of-flight mass spectrometry, UPLC-ESI-QTOF-MS. A total of 31 major compounds were identified in the petal and leaf methanolic extracts from these taxa, utilizing a combination of standards, published data, and fragmentation patterns (Supplementary Table 2). The majority of the compounds identified from the methanolic extracts were acylated and glycosylated flavonoids. Glycosyl groups included glucose, rhamnose, and diglucosides (like sophoroside), many of which have been observed in previous studies (Silva et al., 2004; Steinharter et al., 1986). The major anthocyanins were glycosides of pelargonidin (**5**), petunidin (**9**), and malvidin (**10**), although cyanidin (**6**), delphinidin (**8**), and peonidin (**7**) glycosides were also found. Pelargonidin (**5**) compounds were the dominant anthocyanins in the red-flowered species, *Iochroma gesnerioides* and *I. fuchsioides*, as previously reported (Smith and Rausher, 2011; Smith et al., 2013). Petunidin (**9**) and malvidin (**10**) compounds, followed by delphinidin (**8**), were the predominant anthocyanins in purple-flowered species *Iochroma cyaneum*, *I. australe*, *I. parvifolium*, and *I. grandiflorum*. Cyanidin (**6**) compounds were found in orange *I. edule* flowers, as well as in both red-flowered species. No anthocyanins were found in white-flowered *I. loxense*. Last, small amounts of delphinidin (**8**) compounds were found in *I. cyaneum* leaf samples.

In addition to anthocyanins, glycosides of the flavonols, kaempferol (**2**), quercetin (**3**), and myricetin (**4**), were present. All species produced kaempferol (**2**) and quercetin (**3**) glycosides, but myricetin (**4**) glycosides were only detected in purple-flowered species. Myricetin (**4**) was present in the petals of all purple flowered species and also in leaves for three (*I. cyaneum*, *I. grandiflorum*, and *I. parvifolium*). No flavones were found in petal or leaf tissues. Flavonoid aglycone presence/absence data is reported in Table 1.

Although the majority of the compounds identified in the methanolic extracts were flavonoids, chlorogenic acid (**11**) and four major phenolamide compounds were found in petals and leaves of all species: a bis(dihydrocaffeoyl)-spermidine (**12**), a tris(dihydrocaffeoyl)-spermidine (**13**), and two N-caffeoyl putrescine isomers (**14, 15**) (Supplementary Fig. 1). Phenolamides have been previously found in *I. cyaneum*, as well as other Solanaceae (Sattar et al., 1990). These phenolamides were found in higher concentrations in petals than in leaves. Phenolamides are co-regulated with flavonoids (Outchkourov et al., 2014), which may in part explain their abundance. In addition, putrescine (**14, 15**) is an integral precursor in the biosynthesis of alkaloids, for which the Solanaceae are well known (Dewey and Xie, 2013; Wang et al., 2008).

2.2. Flavonoid quantification with HPLC-UV

HPLC-UV was used to quantify the levels of flavonoid production in the same eight species used for UPLC-MS plus one additional purple-flowering species of Iochrominae (*Vassobia breviflora*) to increase the phylogenetic distance represented in this study. All six common anthocyanidins were detected in hydrolyzed samples using HPLC-UV (Table 1). Delphinidin (**8**) was found in all of the purple flowers, while the other trihydroxylated pigments, petunidin (**9**) and malvidin (**10**), were found in all purple flowers except for *Vassobia brevifolia*. Cyanidin (**6**) was only detected in orange *I. edule* petals. Peonidin (**7**), however, was detected in small quantities in three of the five purple-flowered species. Quercetin (**3**) was the predominant flavonol in both flowers and leaves in all species except for the red-flowered *I. gesnerioides* and *I. fuchsioides*. Generally, the two red-flowered species accumulated lower levels of flavonols than the other species analyzed, with more kaempferol (**2**) than quercetin (**3**) in both tissues (Fig. 2). Quantifiable levels of myricetin (**4**) were only detected in purple-flowered *I. parvifolium* petals. Finally, trace amounts of delphinidin (**8**) were detected in *I. cyaneum* and *V. breviflora* leaves. A comparison of flavonoid aglycone quantities by hydroxylation level from hydrolyzed samples is shown in Fig. 2.

2.3. Principal components analysis of flavonoid production

The phylogenetic principal components analysis (pPCA) partitioned the variance of the HPLC-UV data from hydrolyzed samples into principal components (PCs) that described both flavonoid concentration and composition (Supplementary Table 3). PC1 was strongly correlated with total flavonoid concentration of the samples (Supplementary Fig. 2; Spearman's $r = 0.89$, $p < 0.0001$, $n = 27$) and explained 37% of the total variance. PC1 was the only principal component in which leaf flavonols strongly loaded (0.82 for both mono- and dihydroxylated flavonols, where 1 is the maximum), indicating that most of the flavonoid variation in leaves relates to concentration rather than composition. PC2 and PC3 together describe flavonoid composition, and explain 19% and 14% of the variance, respectively (Fig. 3A). When plotted, PC2 and PC3 scores cluster the analyzed samples into floral color groups, differentiating those from purple-flowering species from those from red-flowering species in particular. White and yellow-flowering samples cluster in the middle, with few to no anthocyanidins detected in hydrolyzed extracts. Together, the PC1-PC3 explained 70.3% of the total variance in the dataset (Supplementary Table 3).

2.4. Correlations among flavonoid hydroxylation groups within tissues

Within-tissue correlations between anthocyanins and flavonols of the same hydroxylation level were predicted, and our analyses revealed such correlations among some types of flavonoids largely within petals (Table 2). Specifically, evidence for correlated production in petal tissue for dihydroxylated and trihydroxylated flavonoids ($r = 0.68$, $r = 0.52$, respectively; Table 2) was found. For example, individuals that produce more trihydroxylated anthocyanins (delphinidin (**8**), petunidin (**9**), and/or malvidin (**10**) glycosides) in their petals also produce more trihydroxylated flavonols (myricetin (**4**) glycosides) in this tissue. This pattern is reflected in the plot of PC loadings where the petal trihydroxylated anthocyanins cluster with their corresponding flavonols (upper left quadrant, Fig. 3B). Comparable correlations among leaf anthocyanins

Please cite this article in press as: Berardi, A.E., et al., Evolutionary correlations in flavonoid production across flowers and leaves in the Iochrominae (Solanaceae), Phytochemistry (2016), http://dx.doi.org/10.1016/j.phytochem.2016.05.007

Table 1
Summary of flavonoids present in petal and leaf tissues. Compounds identified using UPLC-MS are binned by flavonoid aglycone, while compounds detected by HPLC-UV were hydrolyzed and identified as aglycones. Symbol + represents compound presence in both HPLC-UV and UPLC-MS chromatograms, symbol X represents compound presence in HPLC-UV chromatograms only, and symbol * represents compound presence in UPLC-MS chromatograms only.

			Flavonol aglycones			Anthocyanin aglycones					
Floral Color	Hydroxylation: Species	Tissue	Mono Kaempferol[c]	Di Quercetin	Tri Myricetin	Mono Pelargonidin	Di Cyanidin	Di Peonidin	Tri Delphinidin	Tri Petunidin	Tri Malvidin
Purple	*I. australe*	petal	+	+	*			*	+	+	+
		leaf	+	+							
Purple	*I. cyaneum*	petal	+	+	*			+	+	+	+
		leaf	+	+	*				*		
Orange	*I. edule*	petal	+	+			+				
		leaf	+	+							
Red	*I. fuchsioides*	petal	*	*		+	*				
		leaf	+	+							
Red	*I. gesnerioides*	petal	+	*		+	*				
		leaf[b]	*	*							
Purple	*I. grandiflorum*	petal	+	+	*			+	+	+	+
		leaf	*	*	*						
White	*I. loxense*	petal	+	+							
		leaf	+	+							
Purple	*I. parvifolium*	petal	+	+	+			+	+	+	+
		leaf	+	+	X						
Purple	*V. breviflora*[a]	petal	X	X					X		
		leaf	X	X					X		

[a] *V. breviflora* was only analyzed with HPLC-UV.
[b] Trace amounts of anthocyanins were found in *I. gesnerioides* leaves in MS chromatograms.
[c] Compound numbers associated with compounds: kaempferol (**2**), quercetin (**3**), myricetin (**4**), pelargonidin (**5**), cyanidin (**6**), peonidin (**7**), delphinidin (**8**), petunidin (**9**), malvidin (**10**).

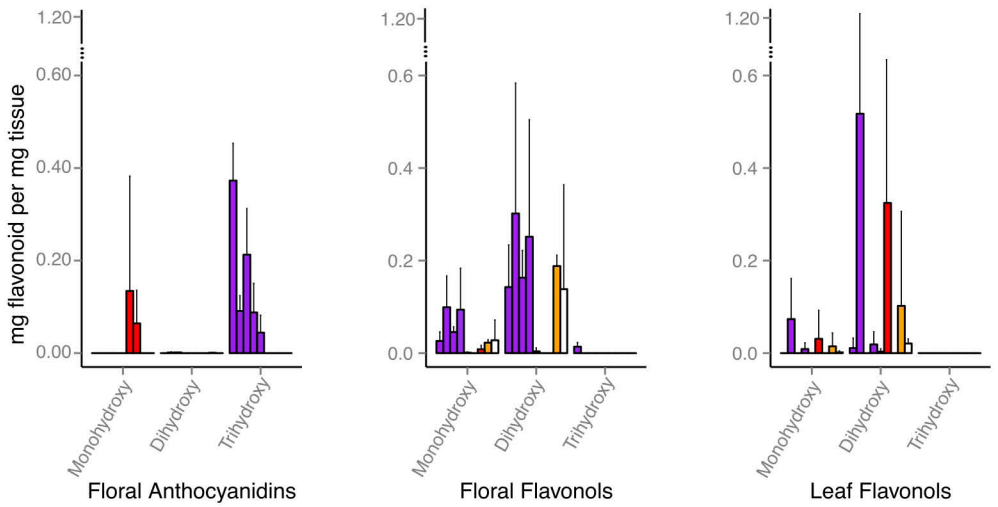

Fig. 2. Species averages of flavonoid aglycone quantities in petals and leaves. Each species is represented by its floral color and in the following order from left to right: purple (*I. parvifolium, I. cyaneum, I. grandiflorum, I. australe, V. breviflora*), red (*I. fuchsioides, I. gesnerioides*), orange (*I. edule*), and white (*I. loxense*). Error bars represent two standard errors of the mean. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and flavonols were not found because leaves largely lacked anthocyanin production (Table 1).

In addition to the expected correlations between flavonoids having the same hydroxylation level, correlations were observed across hydroxylation types within tissues. For example, a strong positive correlation was observed between monohydroxylated and dihydroxylated flavonols in petals ($r = 0.78$; Table 2). Leaves displayed an even stronger correlation between these two classes of flavonols ($r = 0.96$, Table 2). This covariation may be related to the shared regulatory factors controlling their production (Pollastri and Tattini, 2011; Ryan et al., 2002; Stracke et al., 2010).

2.5. Correlations among flavonoid hydroxylation groups between floral and leaf tissues

Few correlations between floral and leaf flavonoids were observed, either within or across hydroxylation levels. Within hydroxylation levels, individuals with higher production of petal dihydroxylated anthocyanins also tended to produce dihydroxylated flavonols in their leaves ($r = 0.60$; Table 2). Across hydroxylation groups, individuals with higher amounts of petal dihydroxylated anthocyanins showed higher amounts of leaf monohydroxylated flavonols ($r = 0.54$, Table 2). Overall, these correlations in flavonoid production across tissues were weaker

Please cite this article in press as: Berardi, A.E., et al., Evolutionary correlations in flavonoid production across flowers and leaves in the Iochrominae (Solanaceae), Phytochemistry (2016), http://dx.doi.org/10.1016/j.phytochem.2016.05.007

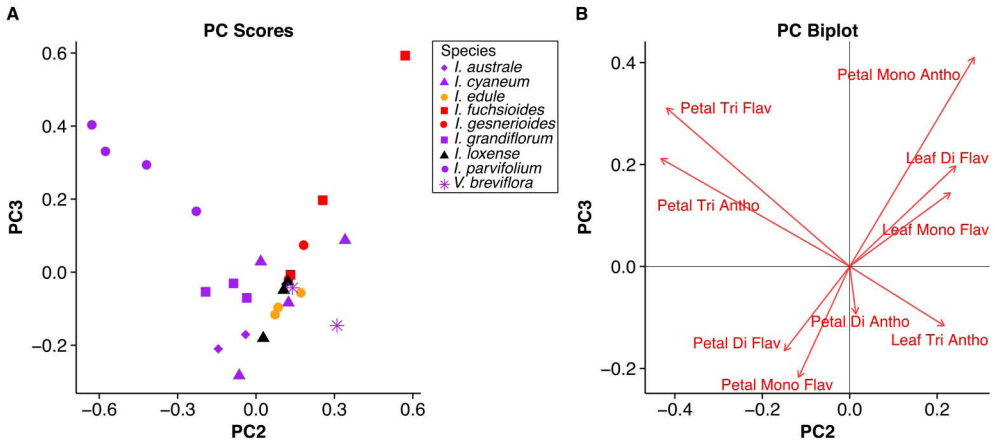

Fig. 3. A: Phylogenetic PC2 and PC3 scores show qualitative differences in the flavonoid profile of the Iochrominae. Species with the same floral color tend to cluster, suggesting that, while controlling for the influence of shared ancestry, species with similar floral color have similar biochemical composition in leaves and petals. B: Biplot of phylogenetic PC2 and PC3 depicting the PC loadings and directions for the quantitative flavonoid dataset. Each variable name has three components: the level of B ring hydroxylation of the flavonoid (monohydroxylated, dihydroxylated, or trihydroxylated as mono, di, or tri), anthocyanin ("Antho") or flavonol ("Flav"), and the tissue (petal or leaf).

Table 2
Phylogenetic correlation matrix generated by pPCA analysis of 26 individuals from nine species. The phylogenetic signal (λ) estimated for the dataset was 0.342. The upper diagonal is bolded for $\alpha = 0.05$ after Bonferroni correction, and the lower diagonal bolded for $\alpha = 0.05$ without the correction. Each variable is labeled by its three components: the tissue type (petal or leaf), the level of hydroxylation of the flavonoid (mono-, di-, or trihydroxylated as mono, di, or tri), and the flavonoid type: anthocyanin or flavonol ("antho" or "flav"). Flavonoids of the same level of hydroxylation are outlined in boxes. Numbers in italics are comparisons within the same tissue. Note that trihydroxylated leaf flavonols were not found.

		Petal Mono Antho	Petal Mono Flav	Leaf Mono Flav	Petal Di Antho	Petal Di Flav	Leaf Di Flav	Petal Tri Antho	Petal Tri Flav	Leaf Tri Antho
Petal	Mono Antho	1	*-0.15*	0.16	*-0.13*	*-0.18*	0.25	*-0.14*	*-0.04*	-0.04
Petal	Mono Flav	*-0.15*	1	0.42	*0.40*	***0.78***	0.29	*0.13*	*0.00*	-0.19
Leaf	Mono Flav	0.16	**0.42**	1	**0.54**	0.44	***0.96***	0.04	-0.05	*-0.05*
Petal	Di Antho	*-0.13*	***0.40***	**0.54**	1	***0.68***	**0.60**	*0.22*	*-0.08*	-0.07
Petal	Di Flav	*-0.18*	***0.78***	**0.44**	***0.68***	1	0.46	*0.22*	*0.13*	-0.20
Leaf	Di Flav	0.25	0.29	***0.96***	**0.60**	**0.46**	1	0.06	-0.03	*-0.04*
Petal	Tri Antho	*-0.14*	*0.13*	0.04	*0.22*	*0.22*	0.06	1	***0.52***	-0.17
Petal	Tri Flav	*-0.04*	*0.00*	-0.05	*-0.08*	*0.13*	-0.03	***0.52***	1	-0.15
Leaf	Tri Antho	-0.04	-0.19	*-0.05*	-0.07	-0.20	*-0.04*	-0.17	-0.15	1

than those observed within tissues.

3. Discussion

This survey of flavonoids in the Iochrominae revealed that the diverse array in flower colors in this clade arises from a similarly diverse set of anthocyanin pigments. Given that the synthesis of different classes of flavonoids is mediated by some of the same enzymatic steps and transcriptional regulators, it can be predicted that this floral variation in anthocyanins would be associated with variation in other flavonoids. Indeed, the analyses here show that changes in floral anthocyanins were significantly correlated with changes in floral flavonols, particularly those with the same B ring hydroxylation pattern (e.g., dihydroxylated anthocyanins and dihydroxylated flavonols). Correlations were also found among flavonoids within leaves in species where both anthocyanins and flavonols were present. Nonetheless, differences in floral flavonoids were only weakly correlated between flowers and leaves.

3.1. Iochrominae flavonoid composition

The survey herein revealed a general correspondence between the color of the flower and the type of anthocyanin present. While the purple-flowered species produce largely or exclusively the 'purple' trihydroxylated delphinidin-based (**8**) anthocyanins, the red-flowered species produce 'red' monohydroxylated pelargonidin-based (**5**) anthocyanins (Table 1; Fig. 2). The form of *I. edule* sampled here contains only cyanidin (**6**) pigments, but appears orange because of the joint expression of yellow carotenoids (Ng and Smith, 2016). Finally, the white-flowered *I. loxense* was found to contain no anthocyanins. This correspondence between flower color and anthocyanin composition is similar to that observed in other Solanaceae (Bar-Akiva et al., 2010; Eich, 2008) and other clades of flowering plants (Wessinger and Rausher, 2012). However, this pattern is not universal. In particular, many red-flowered taxa are colored by di- or trihydroxylated anthocyanins but appear red due to reduced vacuolar pH or the presence of yellow-orange carotenoids (Ando et al., 1999; Beale et al., 1941;

Please cite this article in press as: Berardi, A.E., et al., Evolutionary correlations in flavonoid production across flowers and leaves in the Iochrominae (Solanaceae), Phytochemistry (2016), http://dx.doi.org/10.1016/j.phytochem.2016.05.007

Griesbach et al., 1999; Ng and Smith, 2016).

In addition to anthocyanins, the Iochrominae produced all three types of flavonols, with quercetin (**3**) and kaempferol (**2**) glycosides being the most common. This observation is in accord with previous surveys (Eich, 2008), which have highlighted the rarity of trihydroxylated myricetin-based flavonols (**4**). In the Iochrominae, myricetin (**4**) was present only in the purple flowered species, which also make trihydroxylated anthocyanins. Thus, the presence of myricetin (**4**) glycosides in purple flowers may be a pleiotropic effect of high F3′5′H activity (Fig. 1).

Finally, flavones were not detected in any species, suggesting that this class of flavonoids is not a major component of the metabolite profile of leaves and flowers in the Iochrominae. Flavones appear to be less common in Solanaceae as a whole (Eich, 2008) and were not found in previous flavonoid analysis in *I. gesnerioides* (Alfonso and Kapetanidis, 1994). Nonetheless, Wollenweber et al. (2005) found flavones in the leaf exudate of *I. australe* (note that exudate was not sampled in the current study). Future studies with additional taxa and tissues or using different environmental conditions may uncover greater flavone diversity.

3.2. Within-tissue correlations

The second aim of our studies was to determine whether transitions in floral anthocyanins lead to correlated changes in other floral flavonoids. In particular, it was predicted that shifts between hydroxylation levels in anthocyanins would result in parallel shifts in hydroxylation level for the flavonols. This prediction was supported for the di- and trihydroxylated forms, e.g. species with more trihydroxylated anthocyanins produce higher amounts of trihydroxylated flavonols. However, increases in the monohydroxylated pelargonidin-based (**5**) anthocyanins in red flowered species did not lead to increases in monohydroxylated flavonols (kaempferol derivatives, **2**). This is surprising because red flowered *Iochroma* lack *F3′H* and *F3′5′H* expression (Smith and Rausher, 2011), which should favor the accumulation of kaempferol (**2**) in addition to pelargonidin (**5**). However, both of the red species produce very small amounts of any flavonols in petals (Fig. 2). One possible explanation is the functional specialization of DFR (dihydroflavonol reductase, Fig. 1) involved in the evolution of pelargonidin (**5**) production. In red-flowered *Iochroma* species, DFR has evolved specificity for dihydrokaempferol (DHK, the pelargonidin precursor) over the other dihydroflavonol substrates (Smith et al., 2013). In this process, DFR may have gained the ability to outcompete FLS (flavonol synthase, Fig. 1) for DHK. Additional studies examining both DFR and FLS activity would be needed to test this hypothesis.

In addition to the predicted correlated shifts among anthocyanins and flavonols at the same hydroxylation level, significant correlations were found across hydroxylation levels. For example, species that produce higher amounts of dihydroxylated flavonols (quercetin derivatives, **3**) also have higher levels of monohydroxylated flavonols (kaempferol derivatives, **2**). This may be related to the stepwise structure of the pathway, where the precursor of kaempferol (DHK) must be made in order to produce the precursor of quercetin (DHQ). Given this structure, we would expect that at least some kaempferol (**2**) would be produced whenever quercetin (**3**) in produced, so long as FLS is efficient on both DHK and DHQ as substrates. This pattern of joint production of quercetin (**3**) and kaempferol (**2**) is found in many taxa in addition to *Iochroma* (Berger et al., 2007; Bidel et al., 2015; Griesbach and Asen, 1990; Kuhn et al., 2011).

3.3. Correlated production across tissues

Compared with the strong correlations in flavonoid production within tissues, relatively independent variation between flowers and leaves was observed. Despite major shifts in floral anthocyanin composition associated with flower color transitions, leaf biochemistry was relatively constant across the group (Fig. 2). Most species, with the exception of *V. breviflora*, produce only flavonols in leaves. The mono- and dihydroxylated flavonols (kaempferol and quercetin; **2**, **3**) were found in leaves of all of the species, regardless of the type of anthocyanins or flavonols found in petals (Table 1). This pattern could reflect tissue-specific differences in flavonoid gene regulation, which have been documented in *Iochroma* (Smith and Rausher, 2011) along with many other plant groups (e.g., (Des Marais and Rausher, 2010; Streisfeld and Rausher, 2009)). However, these results may also relate to the specific conditions under which the plants were grown in this study (see Section 5.1). For example, more stressful growing conditions might have led to increased vegetative anthocyanin production (Warren and Mackenzie, 2001) and revealed greater associations between the types of flavonoids produced in leaves and in flowers.

4. Conclusions

Evolutionary studies of flavonoid production have largely focused on changes in floral anthocyanins, which are key contributors to floral hue (Hopkins and Rausher, 2011; Rausher, 2008; Smith and Rausher, 2011; Wessinger and Rausher, 2014; Whittall et al., 2006). This study shows that these shifts in floral anthocyanins can result in correlated changes in floral flavonols, particularly those at the same hydroxylation level. These correlations are predictable based on the structure of the pathway given that the expression of the hydroxylating enzymes (F3′H and F3′5′H) provides precursors for both types of flavonoids. The changes in floral biochemistry did not result in strongly correlated variation in leaf biochemistry, a pattern that may reflect independent regulation of the pathway in these tissues. Nonetheless, flavonoid production is strongly influenced by abiotic and biotic factors, such as UV stress and herbivory (Gaudinier et al., 2015; Strauss and Whittall, 2006). Thus, additional exploration of floral and leaf composition under multiple growing conditions would clarify the generality of this finding.

5. Experimental

5.1. Sample preparation and extraction

Nine species from two genera, *Iochroma* and *Vassobia*, were studied that represent the full range of floral colors produced by the Iochrominae (Supplementary Table 1). Leaf and corolla tissue were collected in September 2014 from greenhouse-grown individuals at the University of Colorado – Boulder (Supplementary Table 1). Plants were grown under natural light in two-gallon pots in Fafard 3 M Mix soil (Conrad Fafard), fertilized weekly with an all-purpose 20-20-20 fertilizer (JR Peters, Inc.), and watered daily or as needed.

All nectar was removed and tissues were patted dry before being placed in individual small plastic bags filled with silica (Flower Drying Art silica gel, #2610, Activa Products Inc.). Samples were dried on silica for approximately 2 weeks at room temperature in darkness. To prepare samples for metabolite profiling, individual dried tissues were removed from the silica, and any remaining powder was brushed off with a small paintbrush. Dried tissues were weighed and ground to fine powders, taking care to not overheat the tissue. One vial of silica gel was also prepared for extraction in order to control for possible silica contaminants in the plant extracts.

In order to determine the range of compounds that were present in the Iochrominae, we employed UPLC-ESI-QTOF-MS. We then

Please cite this article in press as: Berardi, A.E., et al., Evolutionary correlations in flavonoid production across flowers and leaves in the Iochrominae (Solanaceae), Phytochemistry (2016), http://dx.doi.org/10.1016/j.phytochem.2016.05.007

used HPLC-UV on a larger set of species and individuals to quantify known aglycone compounds from hydrolyzed samples. To prepare extracts for both analyses, an aliquot of flavonoid extraction buffer (99% MeOH (Spectrum Chemicals), 1% HCO_2H (Sigma-Aldrich), v/v, MS grade) of 18 μl per mg of dry tissue was added to each sample and vortexed to mix. Samples were centrifuged at 18,000x *g* for 1 min so that all tissue was submerged in the extraction buffer. Samples were sonicated in a water bath for 10 min at room temperature, and then re-centrifuged at 18,000x *g* for 10 min to create a dense pellet of tissue. Two aliquots were taken from each supernatant: 100 μl for UPLC-MS and 200 μl for HPLC-UV. All aliquots were dried under nitrogen gas and stored at −20 °C.

5.2. Qualitative UPLC-MS

Dried extracts were resuspended in MS-grade H_2O containing 0.1% HCO_2H (Sigma-Aldrich, v/v): CH_3CN (Spectrum Chemicals) (100 μl, 90:10 v/v). Samples were sonicated and centrifuged at 10,000x *g* for 10 min to remove particulate matter and insoluble compounds. An aliquot of the supernatant (50 μl) was transferred into an autosampler vial. Petal and leaf "master mixtures" were created by pooling aliquots (2 μl) from each petal and leaf sample, respectively. Untargeted profiling of the extracts was performed using a Waters Acquity I-Class UPLC (Waters Corp.) fitted with a BEH Acquity C18 reversed phase column (2.1 × 50 mm, 1.7 μM) (Waters Corp) and coupled with a Synapt-G2S-HDMS (ESI-QTOF) (Waters Corp.). The binary solvent system was A: H_2O containing 0.1% HCO_2H and B: CH_3CN with 0.1% HCO_2H. The UPLC autosampler sample chamber was kept at 8 °C, column temperature 35 °C, and the flow rate was 0.2 mL/min with an injection volume of 1 μl. A 15 min gradient with a linear ramp was established with the following conditions: 0–1 min 99% A, 1–7 min 99–70% A, 7–12 min 70–5% A, 12:30–13 min hold at 5% A, 13–14:30 min 99% A.

Samples were analyzed in high-resolution MS^E mode in both positive and negative ion mode with data collection in centroid mode. The MS parameters were the same for both modes with the exception of the capillary voltage of 3.0 kV in positive and 2.4 kV in negative mode, 50–1800 m/z scan range, 40 V cone voltage, 80 source offset, 120 °C source temperature, 350 °C desolvation temperature, 500 L/hr desolvation gas flow, 50 L/hr cone gas flow, 6 Bar nebulizer gas flow, low energy 4 transfer/2 trap, high energy ramp 10–45 transfer/2 trap, and a cycle time of 0.2 s for both high and low energy scans. Lockmass (1 ng/mL leucine encephalin (Waters Corp.)) was infused at 5 μl/min and scanned every 20 s with a cycle time of 0.2 s. Each sample was injected twice for technical replication.

Targeted MS^2 was performed to obtain fragmentation patterns of the aglycone flavonoid and anthocyanin compounds to aid in identification of isobaric compounds. The MS^2 parameters were identical to the positive mode conditions above, with the exception of increased cone voltage (65 V) and scan time (0.5 s) designed to increase aglycone formation due to breakage of glycosidic bonds and obtain pseudo-MS^3 analysis of the flavonoid aglycone. Individual runs were performed with both leaf and petal master mixes and data was collected for the entire 15 min period. Target masses for common flavonoid aglycones used were: 271.06 (pelargonidin, apigenin; **5, 16**), 287.06 (cyanidin, kaempferol, luteolin; **6, 2, 17**), 301.07 (peonidin, **7**), 303.05 (delphinidin, quercetin, tricetin; **8, 3, 18**), 317.07 (petunidin, isorhamnetin; **9, 19**), 319.05 (myrcetin, **4**), 331.08 (malvidin, **10**). Collision energies were ramped from 10 to 45 V. The following chemical standards were analyzed with targeted MS^2 parameters for comparison to unknowns: delphinidin (**8**), cyanidin (**6**), pelargonidin (**5**), malvidin (**10**), peonidin (**7**), petunidin (**9**), tricetin (**18**) (Extrasynthese; Genay, France); kaempferol (**2**), quercetin (**3**), myricetin (**4**), isorhamnetin (**9**), chlorogenic acid (**11**) (Sigma Aldrich); luteolin (**17**), and apigenin (**16**) (Cayman Chemical; Michigan, USA). Chemical structures of compounds 11–19 are found in the Supplementary Materials.

Flavonols, flavones, and anthocyanins frequently have the same masses, so compound identification of the flavonoids was performed essentially as described by Abrankó and Szilvássy (2015). A detailed explanation of compound identification is also provided in the Supplementary Materials (Supplementary Figs. 5 and 6). Assignment of the parent masses and glycosylation patterns were performed using the low-energy and high-energy MS^E data, respectively. The high energy fragmentation data provided the aglycone flavonoid or anthocyanin, which were matched to the fragmentation of authentic standards and publicly available spectra: KNaPSacK, MassBank, Metlin, and ReSpect, (Afendi et al., 2012; Horai et al., 2010; Sawada et al., 2012; Smith et al., 2005). The identification of phenolamides was performed through matching MS^E fragmentation patterns to the previously mentioned publicly available databases. In order to determine the distribution of the identified compounds within the Iochrominae samples, chromatograms were imported into MarkerLynx XS (Waters Corp.) for chromatogram alignment, feature identification and determination of peak areas. A chromatogram of the floral "master mixture" is provided in the Supplementary materials (Supplementary Fig. 3). Additionally, chromatograms from the silica gel bead extraction were analyzed qualitatively and it was determined that peaks related to the silica gel did not co-elute with flavonoid or phenolic peaks (Supplementary Fig. 3).

5.3. HPLC-UV analysis of flavonoid aglycones

Hydrolyzing flavonoids can often help in identifying their relative quantities, as there are fewer opportunities for co-chromatographing glycosides and isomers. The major flavonoids identified in the qualitative UPLC-MS were hydrolysable (o-vs c-glycosylated), so the resulting flavonoid aglycones were quantified. The dried aliquots of the methanolic extract that had been used for UPLC-MS were further extracted as outlined in Harborne (1998). Modifications to the Harborne (1998) protocol were made as follows: samples were incubated in screw cap tubes (2 mL) in a dry heating block at 95 °C for 60 min. EtOAc (400 μl × 2) was added to each sample, vortexed, and centrifuged for 1 min at 16,000x *g*. The EtOAc supernatants were removed and evaporated under a stream of air for flavone and flavonol aglycone analysis. Next, isoamyl alcohol (150 μl × 2) was added to the original 2 N HCl extract, vortexed as a whole, and centrifuged as above. The isoamyl alcohol supernatants were then removed and evaporated under a stream of air for anthocyanidin aglycone analysis. Additional Iochrominae biological replicates and one additional species (*Vassobia breviflora*) were collected in the CU Boulder greenhouse in April 2015 to supplement the sample set used for MS analysis (Supplementary Table 1) for a total of nine species and 27 samples. The samples were collected and dried as described above, and pre-soaked overnight in 2 N HCl before extracting with the modified Harborne protocol.

Dried extracts were resuspended in MeOH:HCl (200 μl, 99:1, v/v). Injections (10 μl) were analyzed with an Agilent Infinity 1260 Infinity Series high performance liquid chromatography system with a 100 × 4.6 mm Chromolith Performance RP-18 endcapped column (EMD Chemicals, Darmstadt, Germany). Anthocyanidins were separated by gradient elution at a flow rate of 1.1 mL/min at 30 °C using solvents A (HPLC-grade H_2O containing 2% CF_3CO_2H) and B (1-propanol, 0.1% CF_3CO_2H) with the following protocol: 0–3 min 0% B, 3–8 min 7% B, 8–15 min 13% B, 15–18 min 15% B, 18–19 min 17% B, 19–20 min 27.5% B, 20–21 min 90% B, 21–27 min 95% B, instantaneous decrease to 0% B at 28 min, isocratic at 0% B

Please cite this article in press as: Berardi, A.E., et al., Evolutionary correlations in flavonoid production across flowers and leaves in the Iochrominae (Solanaceae), Phytochemistry (2016), http://dx.doi.org/10.1016/j.phytochem.2016.05.007

from 28 to 30 min. Peaks were detected at 520 and 540 nm with a photo diode array detector (PDA), scanning from 200 to 600 nm at a step of 2 nm. Anthocyanidins were identified by comparison with standard solutions of delphinidin (**8**), cyanidin (**6**), pelargonidin (**5**), malvidin (**10**), peonidin (**7**), and petunidin (**9**) from Extrasynthese (Genay, France).

Non-anthocyanidin flavonoids (flavonols, flavones, etc.) were separated following Repollés et al. (2006) with a few modifications. We used a gradient elution at a flow rate of 2.0 mL/min at room temperature using solvents A (H_2O with 0.1% CF_3CO_2H), B (MeOH with 0.05% CF_3CO_2H) and C (CH_3CN) with the following protocol: starting conditions were 75% A, 25% B, 0–1.5 min 57.5% A, 35% B, 7.5% C, 1.5–9 min 60% A, 40% B, 9–15 min 40% A, 60% B, returning to starting conditions after 15 min. Peaks were detected at 365 nm, with the PDA scanning from 190 to 400 nm at a step of 2 nm. Flavonols and flavones were identified by comparison with standard solutions of kaempferol (**2**), quercetin (**3**), myricetin (**4**) (Sigma Aldrich), luteolin (**17**), apigenin (**16**) (Cayman Chemical; Michigan, USA), and tricetin (**18**) (Extrasynthese; Genay, France).

Chromatograms were visualized using Agilent ChemStation for LC 3D Systems (Rev. B0403, Waldbronn, Germany). Peaks were identified by comparison to retention time and UV spectra of chemical standards. Quantification of each compound was conducted by creating a dilution series of a standard compound of each flavonoid aglycone (the same standards were used from the MS experiment). Peaks were integrated in ChemStation using peak area, and compared to the generated standard curve. Concentrations were then converted to mg of flavonoid to mg of dried tissue.

5.4. Comparative and correlative analyses among hydroxylation levels of flavonoid compounds

To assess associations among flavonoid hydroxylation levels, aglycone compound concentrations were binned as monohydroxylated, dihydroxylated, or trihydroxylated compounds per class (anthocyanidin or flavonol) and per sample. The effects of shared ancestry on phenotypic variance were controlled for by taking a comparative approach to the analysis of multiple species. A previously published three-gene phylogeny of the Iochrominae (Muchhala et al., 2014) was pruned to the nine taxa in this study in R v3.2.0 (Team, 2015) using phytools (Revell, 2012). Individual-level variation was incorporated by splitting each tip (species) into a polytomy with one short branch (1/100 the length of the subtending branch) for each sampled individual (Supplementary Table S1). Phylogenetic principal components analysis (pPCA) was then used to determine whether the quantitative flavonoid aglycone profiles of the Iochrominae petals and leaves clustered by floral color, and to assess correlations among compounds across species (Ackerly and Donoghue, 1998). Specifically, the "phyl.PCA" function in phytools was used on the leaf and petal flavonoid aglycone concentrations for each sample with eigenvalue decomposition of the correlation matrix and phylogenetic signal estimation using Pagel's λ (Pagel, 1999). Optimizing λ, instead of using a Brownian motion model, is recommended for pPCA when there are strong correlations among traits (as is often the case with metabolomic data) (Uyeda et al., 2015). The phylogenetic correlation matrix from the pPCA was examined for broad patterns of significant positive or negative correlations among the compounds in the data. The pPCA results were qualitatively examined using biplots of the first three PCs. Correlations among the flavonoid hydroxylation groups were examined within and among tissues by referencing the phylogenetically corrected correlation matrix generated by the pPCA.

Acknowledgements

This work was supported by NSF IOS #0955517 EDEN Research Exchange Award to A. Berardi, and NSF DEB #1355518 to S. D. Smith. The authors also gratefully acknowledge the support of the Virginia Tech Mass Spectrometry Incubator by the Fralin Life Science Institute, as well as assistance from Eva Collakova and Jody Jervis.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.phytochem.2016.05.007.

References

Abrankó, L., Szilvássy, B., 2015. Mass spectrometric profiling of flavonoid glycoconjugates possessing isomeric aglycones. J. Mass Spectrom. 50, 71–80.
Ackerly, D.D., Donoghue, M.J., 1998. Leaf size, sapling allometry, and Corner's rules: phylogeny and correlated evolution in maples (*Acer*). Am. Nat. 152, 767–791.
Afendi, F.M., Okada, T., Yamazaki, M., Hirai-Morita, A., Nakamura, Y., Nakamura, K., Ikeda, S., Takahashi, H., Altaf-Ul-Amin, M., Darusman, L.K., Saito, K., Kanaya, S., 2012. KNApSAcK family databases: integrated metabolite-plant species databases for multifaceted plant research. Plant & Cell Physiol. 53 e1–e1.
Albert, N.W., Davies, K.M., Lewis, D.H., Zhang, H., Montefiori, M., Brendolise, C., Boase, M.R., Ngo, H., Jameson, P.E., Schwinn, K.E., 2014. A conserved network of transcriptional activators and repressors regulates anthocyanin pigmentation in eudicots. Plant Cell 26, 962–980.
Alfonso, D., Kapetanidis, I., 1994. Flavonoids from *Iochroma gesnerioides*. Pharm. Acta Helv. 68, 211–214.
Ando, T., Saito, N., Tatsuzawa, F., Kakefuda, T., Yamakage, K., Ohtani, E., Koshiishi, M., Matsusake, Y., Kokubun, H., Watanabe, H., Tsukamoto, T., Ueda, Y., Hashimoto, G., Marchesi, E., Asakura, K., Hara, R., Seki, H., 1999. Floral anthocyanins in wild taxa of *Petunia* (Solanaceae). Biochem. Syst. Ecol. 27, 623–650.
Armbruster, W.S., 2002. Can indirect selection and genetic context contribute to trait diversification? A transition-probability study of blossom-colour evolution in two genera. J. Evol. Biol. 15, 468–486.
Bar-Akiva, A., Ovadia, R., Rogachev, I., Bar-Or, C., Bar, E., Freiman, Z., Nissim-Levi, A., Gollop, N., Lewinsohn, E., Aharoni, A., Weiss, D., Koltai, H., Oren-Shamir, M., 2010. Metabolic networking in *Brunfelsia calycina* petals after flower opening. J. Exp. Bot. 61, 1393–1403.
Beale, G.H., Price, J.R., Sturgess, V.C., 1941. A survey of anthocyanins. VII. The natural selection of flower colour. Proc. R. Soc. Lond. B Biol. Sci. 130, 113–126.
Berardi, A.E., 2014. The Role of the Plant Flavonoid Pathway in Adaptation to Elevation [Ph.D. Dissertation]. Department of Biology. University of Virginia, Charlottesville, VA, USA, p. 201.
Berger, J.M., Itagaki, Y., Nakanishi, K., 2007. The effect of ultraviolet-depleted light on the flavonol contents of the cactus species *Opuntia wilcoxii* and *Opuntia violacea*. Chem. Biodivers. 4, 1525–1532.
Bidel, L.P.R., Chomicki, G., Bonini, F., Mondolot, L., Soulé, J., Coumans, M., La Fisca, P., Baissac, Y., Petit, V., Loiseau, A., Cerovic, Z.G., Gould, K.S., Jay-Allemand, C., 2015. Dynamics of flavonol accumulation in leaf tissues under different UV-B regimes in *Centella asiatica* (Apiaceae). Planta 242, 545–559.
Chalker-Scott, L., 1999. Environmental significance of anthocyanins in plant stress responses. Photochem. Photobiol. 70, 1–9.
Chen, Y., Lübberstedt, T., 2010. Molecular basis of trait correlations. Trends Plant Sci. 15, 454–461.
De Jong, W.S., Eannetta, N.T., De Jong, D.M., Bodis, M., 2004. Candidate gene analysis of anthocyanin pigmentation loci in the Solanaceae. Theor. Appl. Genet. 108, 423–432.
Des Marais, D.L., Rausher, M.D., 2010. Parallel evolution at multiple levels in the origin of hummingbird pollinated flowers in *Ipomoea*. *Evolution* 64, 2044–2054.
Dewey, R.E., Xie, J., 2013. Molecular genetics of alkaloid biosynthesis in *Nicotiana tabacum*. Phytochemistry 94, 10–27.
Eich, E., 2008. Solanaceae and Convolvulaceae: Secondary Metabolites. Springer, Berlin, p. 637.
Gaudinier, A., Tang, M., Kliebenstein, D.J., 2015. Transcriptional networks governing plant metabolism. Curr. Plant Biol. 1–9.
Goss, J.A., 1973. Physiology of Plants and Their Cells: Pergamon Biological Sciences Series. Elsevier Science, p. 474.
Griesbach, R.J., Asen, S., 1990. Characterization of the flavonol glycosides in Petunia. Plant Sci. 70, 49–56.
Griesbach, R.J., Stehmann, J.R., Meyer, F., 1999. Anthocyanins in the "red" flowers of *Petunia exserta*. Phytochemistry 51, 525–528.
Gronquist, M., Bezzerides, A., Attygalle, A., Meinwald, J., Eisner, M., Eisner, T., 2001. Attractive and defensive functions of the ultraviolet pigments of a flower (*Hypericum calycinum*). Proc. Natl. Acad. Sci. U. S. A. 98, 13745–13750.
Halbwirth, H., 2010. The creation and physiological relevance of divergent hydroxylation patterns in the flavonoid pathway. Int. J. Mol. Sci. 11, 595–621.
Harborne, J.B., 1977. Flavonoids and the evolution of the angiosperms. Biochem.

Syst. Ecol. 5, 7–22.
Harborne, J.B., 1993. Introduction to Ecological Biochemistry. Academic Press, p. 384.
Harborne, J.B., 1998. Phytochemical Methods: a Guide to Modern Techniques of Plant Analysis. Chapman & Hall, London, p. 302.
Hopkins, R., Rausher, M.D., 2011. Identification of two genes causing reinforcement in the Texas wildflower *Phlox drummondii*. Nature 469, 411–414.
Horai, H., Arita, M., Kanaya, S., Nihei, Y., Ikeda, T., Suwa, K., Ojima, Y., Tanaka, K., Tanaka, S., Aoshima, K., 2010. MassBank: a public repository for sharing mass spectral data for life sciences. J. mass Spectrom. 45, 703–714.
Jeong, H., Tombor, B., Albert, R., Oltvai, Z.N., Barabási, A.L., 2000. The large-scale organization of metabolic networks. Nature 407, 651–654.
Koes, R.E., Spelt, C.E., Mol, J.N., 1989. The chalcone synthase multigene family of *Petunia hybrida* (V30): differential, light-regulated expression during flower development and UV light induction. Plant Mol. Biol. 12, 213–225.
Koski, M.H., Ashman, T.-L., Campbell, D., 2014. Dissecting pollinator responses to a ubiquitous ultraviolet floral pattern in the wild. Funct. Ecol. 28, 868–877.
Kuhn, B.M., Geisler, M., Bigler, L., Ringli, C., 2011. Flavonols accumulate asymmetrically and affect auxin transport in Arabidopsis. Plant Physiol. 156, 585–595.
Lawrence, W.J.C., Price, J.R., Robinson, G.M., Robinson, R., 1939. The distribution of anthocyanins in flowers, fruits and leaves. Philos. Trans. R. Soc. Lond. B Biol. Sci. 230, 149–178.
Lee, D.W., Gould, K.S., 2002. Anthocyanins in leaves and other vegetative organs: an introduction. Adv. Bot. Res. 37, 1–16.
Lewis, C.E., Walker, J.R.L., Lancaster, J.E., Sutton, K.H., 1998. Determination of anthocyanins, flavonoids and phenolic acids in potatoes. I: coloured cultivars of *Solanum tuberosum* L. J. Sci. Food Agric. 77, 45–57.
Lowry, D.B., Sheng, C.C., Lasky, J.R., Willis, J.H., 2012. Five anthocyanin polymorphisms are associated with an R2R3-MYB cluster in *Mimulus guttatus* (Phrymaceae). Am. J. Bot. 99, 82–91.
Martin, C., Prescott, A., Mackay, S., Bartlett, J., Vrijlandt, E., 1991. Control of anthocyanin biosynthesis in flowers of *Antirrhinum majus*. Plant J. 1, 37–49.
Muchhala, N., Johnsen, S., Smith, S.D., 2014. Competition for hummingbird pollination shapes flower color variation in Andean Solanaceae. Evolution 68, 2275–2286.
Ng, J., Smith, S.D., 2016. Widespread flower color convergence in Solanaceae via alternate biochemical pathways. New Phytol. 209, 407–417.
Otto, S.P., 2004. Two steps forward, one step back: the pleiotropic effects of favoured alleles. Proc. R. Soc. B Biol. Sci. 271, 705–714.
Outchkourov, N.S., Carollo, C.A., Gomez-Roldan, V., de Vos, R.C.H., Bosch, D., Hall, R.D., Beekwilder, J., 2014. Control of anthocyanin and non-flavonoid compounds by anthocyanin-regulating MYB and bHLH transcription factors in *Nicotiana benthamiana* leaves. Front. Plant Sci. 5, 519–519.
Pagel, M., 1999. Inferring the historical patterns of biological evolution. Nature 401, 877–884.
Pichersky, E., Gang, D.R., 2000. Genetics and biochemistry of secondary metabolites in plants: an evolutionary perspective. Trends Plant Sci. 5, 439–445.
Pollastri, S., Tattini, M., 2011. Flavonols: old compounds for old roles. Ann. Bot. 108, 1225–1233.
Quattrocchio, F., Wing, J., van der Woude, K., Souer, E., de Vetten, N., Mol, J., Koes, R., 1999. Molecular analysis of the *anthocyanin2* gene of *Petunia* and its role in the evolution of flower color. Plant Cell 11, 1433–1444.
Ramsay, N.A., Glover, B.J., 2005. MYB-bHLH-WD40 protein complex and the evolution of cellular diversity. Trends Plant Sci. 10, 63–70.
Rausher, M.D., 2008. Evolutionary transitions in floral color. Int. J. Plant Sci. 169, 7–21.
Ravasz, E., Somera, A.L., Mongru, D.A., Oltvai, Z.N., Barabási, A.-L., 2002. Hierarchical organization of modularity in metabolic networks. Science 297, 1551–1555.
Repollés, C., Herrero-Martínez, J.M., Ràfols, C., 2006. Analysis of prominent flavonoid aglycones by high-performance liquid chromatography using a monolithic type column. J. Chromatogr. A 1131, 51–57.
Revell, L.J., 2012. phytools: an R package for phylogenetic comparative biology (and other things). Methods Ecol. Evol. 3, 217–223.
Ryan, K.G., Swinny, E.E., Markham, K.R., Winefield, C., 2002. Flavonoid gene expression and UV photoprotection in transgenic and mutant *Petunia* leaves. Phytochemistry 59, 23–32.
Sattar, E.A., Glasl, H., Nahrstedt, A., Hilal, S.H., 1990. Hydroxycinnamic acid amides from *Iochroma cyaneum*. Phytochemistry 29, 3931–3933.
Sawada, Y., Nakabayashi, R., Yamada, Y., Suzuki, M., Sato, M., Sakata, A., Akiyama, K., Sakurai, T., Matsuda, F., Aoki, T., 2012. RIKEN tandem mass spectral database (ReSpect) for phytochemicals: a plant-specific MS/MS-based data resource and database. Phytochemistry 82, 38–45.
Schilling, C.H., Palsson, B.O., 1998. The underlying pathway structure of biochemical reaction networks. Proc. Natl. Acad. Sci. U. S. A. 95, 4193–4198.
Silva, T.M.S., Nascimento, R.J.B., Câmara, C.A., Castro, R.N., Braz-Filho, R., Agra, M.D.F., Geraldo de Carvalho, M., 2004. Distribution of flavonoids and n-trans-caffeoyltyramine in *Solanum* subg. *Leptostemonum*. Biochem. Syst. Ecol. 32, 513–516.
Smith, S.D., Baum, D.A., 2006. Phylogenetics of the florally diverse andean clade Iochrominae (Solanaceae). Am. J. Bot. 93, 1140–1153.
Smith, S.D., Rausher, M.D., 2011. Gene loss and parallel evolution contribute to species difference in flower color. Mol. Biol. Evol. 28, 2799–2810.
Smith, C.A., O'Maille, G., Want, E.J., Qin, C., Trauger, S.A., Brandon, T.R., Custodio, D.E., Abagyan, R., Siuzdak, G., 2005. METLIN: a metabolite mass spectral database. Ther. Drug Monit. 27, 747–751.
Smith, S.D., Wang, S., Rausher, M.D., 2013. Functional evolution of an anthocyanin pathway enzyme during a flower color transition. Mol. Biol. Evol. 30, 602–612.
Steinharter, T.P., Cooper-Driver, G.A., Anderson, G.J., 1986. The phylogenetic relationship of *Solanum* flavonols. Biochem. Syst. Ecol. 14, 299–305.
Stracke, R., Ishihara, H., Huep, G., Barsch, A., Mehrtens, F., Niehaus, K., Weisshaar, B., 2007. Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the *Arabidopsis thaliana* seedling. Plant J. 50, 660–677.
Stracke, R., Jahns, O., Keck, M., Tohge, T., Niehaus, K., Fernie, A.R., Weisshaar, B., 2010. Analysis of PRODUCTION OF FLAVONOL GLYCOSIDES-dependent flavonol glycoside accumulation in *Arabidopsis thaliana* plants reveals MYB11-, MYB12- and MYB111-independent flavonol glycoside accumulation. New Phytol. 188, 985–1000.
Strauss, S.Y., Whittall, J.B., 2006. Non-pollinator agents of selection on floral traits. In: Harder, L.D., Barrett, S.C.H. (Eds.), Ecology and Evolution of Flowers. Oxford University Press, Oxford, pp. 120–138.
Streisfeld, M.A., Rausher, M.D., 2009. Altered trans-regulatory control of gene expression in multiple anthocyanin genes contributes to adaptive flower color evolution in *Mimulus aurantiacus*. Mol. Biol. Evol. 26, 433–444.
Taylor, T.W.J., 1940. Plant pigments in the Galapagos Islands. Proc. R. Soc. Lond. B Biol. Sci. 129, 230–237.
Team, R.C., 2015. R: A Language and Environment for Statistical Computing (Vienna).
Uyeda, J.C., Caetano, D.S., Pennell, M.W., 2015. Comparative analysis of principal components can be misleading. Syst. Biol. 0, 1–13.
van Tunen, A.J., Koes, R.E., Spelt, C.E., van der Krol, A.R., Stuitje, A.R., Mol, J.N., 1988. Cloning of the two chalcone flavanone isomerase genes from *Petunia hybrida*: coordinate, light-regulated and differential expression of flavonoid genes. EMBO J. 7, 1257–1263.
Wang, P., Liang, Z., Zeng, J., Li, W., Sun, X., Miao, Z., Tang, K., 2008. Generation of tobacco lines with widely different reduction in nicotine levels via RNA silencing approaches. J. Biosci. 33, 177–184.
Warren, J., Mackenzie, S., 2001. Why are all colour combinations not equally represented as flower-colour polymorphisms? New Phytol. 151, 237–241.
Wessinger, C.A., Rausher, M.D., 2012. Lessons from flower colour evolution on targets of selection. J. Exp. Bot. 63, 5741–5749.
Wessinger, C.A., Rausher, M.D., 2014. Predictability and irreversibility of genetic changes associated with flower color evolution in *Penstemon barbatus*. Evolution 68, 1058–1070.
Whittall, J.B., Voelckel, C., Kliebenstein, D.J., Hodges, S.A., 2006. Convergence, constraint and the role of gene expression during adaptive radiation: floral anthocyanins in *Aquilegia*. Mol. Ecol. 15, 4645–4657.
Winkel-Shirley, B., 1996. Flavonoid biosynthesis: 'new' functions for an 'old' pathway. Trends Plant Sci. 1, 377–382.
Wollenweber, E., Dörsam, M., Dörr, M., Roitman, J.N., Valant-Vetschera, K.M., 2005. Chemodiversity of surface flavonoids in Solanaceae. Z. Naturforschung. – Sect. C J. Biosci. 60, 661–670.
Xi, Y., Chen, Y.P., Qian, C., Wang, F., 2011. Comparative study of computational methods to detect the correlated reaction sets in biochemical networks. Brief Bioinform. 12, 132–150.

Please cite this article in press as: Berardi, A.E., et al., Evolutionary correlations in flavonoid production across flowers and leaves in the Iochrominae (Solanaceae), Phytochemistry (2016), http://dx.doi.org/10.1016/j.phytochem.2016.05.007