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Lessons from flower colour evolution on targets of selection

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Abstract

The genetic basis of flower colour evolution provides a useful system to address the debate over the relative contribution of regulatory vs. functional mutations in evolution. The relative importance of these two categories depends on the type of flower colour transition and the genes involved in those transitions. These differences reflect differences in the degree of deleterious pleiotropy associated with functional inactivation of various anthocyanin pathway genes. Our findings illustrate how generalized statements regarding the contributions of regulatory and functional mutations to broad categories of traits, such as morphological vs. physiological, ignore differences among traits within categories and in doing so overlook important factors determining the relative importance of regulatory and functional mutations.

Key words: Adaptive evolution, adaptive mutation, anthocyanins, flower colour, pleiotropy.

Introduction

Novel phenotypes are ultimately derived from mutations that are favoured by natural selection. An important goal of evolutionary genetics is to understand, and possibly predict, whether certain types of mutations are preferentially involved in evolutionary change. Only recently, with the widespread availability of molecular genetic and genomic tools, has it become possible to address this goal. Documentation of genetic changes involved with phenotypic change in diverse taxa is accumulating (for summaries see Hoekstra and Coyne, 2007; Stern and Orgogozo, 2008). These data have sparked a vigorous scientific dialogue regarding the nature of genetic mutations that contribute to evolutionary change (Stern, 2000; Hoekstra and Coyne, 2007; Wray, 2007; Carroll, 2008; Stern and Orgogozo, 2008; Wagner and Lynch, 2008; Streifeld and Rausher, 2010). This debate centres largely on whether phenotypic changes proceed primarily through regulatory changes or primarly through changes to coding sequences that affect protein function, and whether the answer to this question depends on the type of trait involved. Broad comparisons across many taxa (e.g. Stern and Orgogozo, 2008) have suggested that the evolution of morphological traits proceeds primarily through regulatory mutations, while the evolution of physiological traits more often involves functional mutations to coding sequences.

The standard explanation offered for this pattern is that morphological and physiological traits differ categorically in their position within developmental pathways (Hoekstra and Coyne, 2007; Stern and Orgogozo, 2008). It is argued that because morphological traits are specified by genes that act upstream in developmental pathways, regulatory changes with subtle effects on gene action in a restricted set of tissues are favoured because they minimize adverse disruption to development. Conversely, since physiological traits are specified by genes near the tips of developmental pathways, functional mutations that occur more frequently than regulatory mutations will by nature have a more limited scope of action and be tolerated. However, Hoekstra and Coyne (2007) point out that the categorization of traits as ‘morphological’ vs. ‘physiological’ is somewhat artifical, and that we need an unbiased framework for understanding when we should predict regulatory or functional mutations to predominantly contribute to evolutionary change in a given trait. In particular, we need to statistically determine whether the evolution of a trait occurs through preferential fixation of certain mutations over alternatives. This endeavour requires the intensive study of genetic changes involved in multiple evolutionary occurrences of individual traits (Kopp, 2009; Streifeld and Rausher, 2010). Yet in the broad surveys that have been
conducted to date, few traits are represented by more than one mutation. Flower colour is a trait that affords an unusual opportunity to adopt this approach. Flower colour is evolutionarily labile, with examples of evolutionary divergence between closely related species from diverse genera and families (Fig. 1). This offers the opportunity to examine ‘replicate’ cases of the same phenotypic change, allowing a rigorous statistical assessment of patterns. In addition, the structure of the anthocyanin biosynthetic pathway, which produces the most important and widespread floral pigments, is conserved across angiosperms and is well-characterized genetically. Because this pathway consists of relatively few enzyme-coding genes and associated regulatory genes (Fig. 2), a candidate-gene approach to dissecting the genetic underpinnings of flower colour divergence has been successful.

Although flower colour divergence may occur through neutral drift or linkage to pleiotropic traits experiencing selection, in many cases such change is likely adaptive. Indirect evidence for a common role of natural selection in flower colour change is provided by the widespread convergent evolution of pollination syndromes (Fenster et al., 2004). Evolutionary shifts from one pollination syndrome to another often involve particular flower colour transitions. For example, shifts from bee to hummingbird pollination are typically accompanied by shifts from blue/purple to red flowers, while shifts from either bee or bird to hawkmoth pollination are often accompanied by shifts from pigmented to pale or white flowers (reviewed by Rausher, 2008). This regularity is inconsistent with genetic drift as major cause of evolutionary change in flower colour. More direct evidence indicating that changes in flower colour are adaptive is provided by a growing body of investigations directly demonstrating the operation of natural selection on flower colour variation (reviewed by Rausher, 2008; Waser and Price, 1981; Jones and Reithel, 2001; Schemske and Bierzychudek, 2001, 2007; Irwin and Strauss, 2005; Streisfeld and Kohn, 2007; Hopkins and Rausher, 2012).

Here we review recent progress using the anthocyanin pathway as a model system for understanding when and why we can predict regulatory or functional genetic changes to predominate in the evolution of flower colour.

**Mutation rate and pleiotropy**

For any given trait, two factors are likely to influence the relative contribution of regulatory and functional mutations to adaptive evolution: the relative rates of different mutations and their relative fitness effects. In the absence of fitness differences among various mutations, mutations that arise more frequently should predominantly contribute to adaptive divergence. The relative mutation rates for regulatory vs. functional mutations will depend on the evolutionarily relevant mutational ‘target size’ of each. This target size will reflect: (1) the number of genes in each category; and (2) the number of nucleotide sites per gene that, if mutated, can produce a particular phenotype, which may be correlated with both the gene length and functional structure of the gene. Target size will also likely depend on whether the new phenotype involves a gain or loss of molecular function.

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**Fig. 1.** Examples of commonly observed evolutionary shifts in flower colour. (A, B) Transition from pigmented *Ipomoea cordatotriloba* to white *I. lacunosa*. (C, D) Transition from purple *Penstemon neomexicanus* to red *P. barbatus*. (A, B) With permission from Tanya Duncan; (C, D) Taken by Carolyn A. Wessinger.
Loss-of-function phenotypes may have a larger mutational target size than gain-of-function phenotypes since there are normally many more nucleotide sites that, if mutated, eliminate a molecular function vs. those that create a novel function.

Any deleterious pleiotropic effects on fitness will negatively influence a mutation’s relative contribution to adaptation. Thus, even if regulatory and functional mutations produce the same advantageous phenotype and enjoy the same fitness benefit, they may have unequal net fitnesses if one negatively perturbs other pleiotropic traits specified by that gene (Otto, 2004). Since the fixation probability for an advantageous mutation is proportional to its net fitness, mutations with minimal deleterious pleiotropic effects will disproportionately contribute to adaptive divergence.

For most traits, little information is available on either relative mutation rates or the relative magnitudes of deleterious pleiotropy for regulatory vs. functional mutations. However, we can obtain an approximate estimate of the rates for regulatory and functional mutations causing particular flower colour shifts because spontaneous flower colour mutants are highly visible and are often preserved by horticulturalists. Although there is little empirical evidence on differential pleiotropy, we can make strong conjectures about the relative pleiotropic effects of different types of mutations based on our understanding of the structure and function of the anthocyanin pathway. This information permits a preliminary evaluation of how relative mutation rates and differential pleiotropy affect the relative contribution of regulatory and functional mutations to evolutionary changes in flower colour.

Fig. 2. The anthocyanin pathway. The pathway has three branches. The anthocyanins produced, and hence flower colour, are determined by which branches are functional. Anthocyanins derived from pelargonidin are red. Those derived from delphinidin tend to be blue/purple. Those derived from cyanidin are variable, from red to magenta to blue/purple, depending on plant taxon. Flux down the different branches is determined largely by the activity of enzymes F3’$h$ and F3’S$'h$. Downregulation or functional inactivation of these enzymes causes flux to be redirected down a different branch, resulting in a different flower colour, as indicated by flower symbols. Production of non-pigmented flowers can be accomplished by downregulation or functional inactivation of other enzymes or transcription factors (marked with ∅). Side branches leading to other flavonoids are in grey. ANS, Anthocyanidin synthase; bHLH, basic-helix-loop-helix; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol-4-reductase; DHK, dihydrokaempferol; DHM, dihydromyricetin; DHQ, dihydroquercetin; F3H, flavanone 3-hydroxylase; F3’S$’h$, flavanoid 3’-hydroxylase; F3’S$’h$, flavanoid 3’,5’-hydroxylase; FLS, flavonol synthase; LAR, leucoanthocyanidin reductase; WDR, WD-repeat.
Evolutionary loss of pigmentation

A flower colour transition common to many angiosperm taxa is loss of floral anthocyanins, which typically produces white, or sometimes yellow, flowers (Fig. 1). Such evolutionary shifts often accompany a switch in pollinators from bees or hummingbirds to moths or bats (Baker, 1963; Stebbins, 1970; Grant, 1994). In theory, shifts from pigmented to white flowers could involve any mutation that blocks one or more steps of the anthocyanin pathway (Fig. 2). These include: (1) loss-of-function (LOF) mutations to any pathway enzyme; (2) cis-regulatory mutations that downregulate any pathway enzyme; (3) LOF to any of the three proteins (R2R3 MYB, bHLH, and WDR) that regulate expression of the enzyme-coding genes; and (4) cis-regulatory mutations that downregulate any of these regulators. For our purposes, we consider mutations in category 1 to be functional and those in categories 2–4 to be regulatory.

Characterization of spontaneous white flower mutants in *Petunia, Antirrhinum, Ipomoea*, and other plant taxa have demonstrated roughly equal frequencies of both regulatory and functional mutations: 29 of 69 tabulated cases involve spontaneous functional mutations to core pathway enzymes (Fig. 2); 8 of 69 cases involved spontaneous cis-regulatory mutations to core pathways enzymes; and 32 of 69 cases involved spontaneous transcription factor mutations (for details, see Streisfeld and Rausher, 2010). By contrast, the evolutionary fixation of white flowers within natural populations or species all involve inactivation of anthocyanin transcription factors (8–12 cases; Streisfeld and Rausher, 2010). This pattern is strikingly and statistically different from that seen in the spontaneous mutations, indicating that mutations affecting transcription regulatory proteins are preferentially fixed in the evolutionary loss of pigmentation. Moreover, in all but one case (where the identity of the regulatory gene is unknown), the mutation fixed by natural selection inactivated the floral R2R3MYB transcription factor. By contrast, most spontaneous regulatory mutations eliminating floral anthocyanins inactivated the bHLH and WDR regulatory proteins. Thus, despite larger mutational target sizes for *bHLH* and *WDR*, mutations to R2R3*Myb* are preferentially fixed (Streisfeld and Rausher, 2010).

This pattern can be explained by differences among these genes in the inferred magnitude of their associated pleiotropy, which we can deduce from properties of the anthocyanin pathway. This pathway, including enzymes and regulatory proteins, is also responsible for producing related classes of flavonoid compounds that perform diverse functions in vegetative plant tissues (Winkel-Shirley, 2002). Most plants deploy a number of R2R3MYB transcription factors to regulate anthocyanin production, each with a tissue-specific or context-dependent expression profile (Ramsay and Glover, 2005; Albert et al., 2011). Consequently, inactivation of the R2R3MYB responsible for regulating anthocyanin production in floral tissues eliminates anthocyanins in that tissue only. By contrast, enzymes of the anthocyanin pathway, as well as the bHLH and WDR regulators, are typically expressed in vegetative tissues as well as floral tissue. LOF mutations to these proteins would not only produce white flowers, but also would block production of other flavonoid compounds that enhance fitness. This is expected to produce deleterious pleiotropic effects, while few such effects are expected for the inactivation of the R2R3MYB. The apparent exclusive involvement of the floral R2R3MYB in evolutionary shifts to white flowers is thus consistent with the expectation that mutations incurring the least deleterious pleiotropy will be preferentially fixed by selection.

We lack extensive empirical data demonstrating the importance of this pleiotropy on plant fitness. However, our limited data are consistent with this interpretation. In field experiments, Rausher and Fry (2003) detected no deleterious pleiotropic fitness effects of an LOF mutation in the floral R2R3MYB gene in *Ipomoea purpurea*. By contrast, an LOF mutation to Chs in *I. purpurea* reduces survival and fecundity substantially (Coberly and Rausher, 2003, 2008), as does an LOF mutation to *Ans* in *Phlox drummondii* (Levin and Brack, 1995; Hopkins and MD Rausher, unpublished). These results support the contention that inactivation of the R2R3MYB protein has a much smaller detrimental pleiotropic effect on fitness than inactivation of enzyme-coding genes. However more experiments of this type with other pathway genes, including the other regulatory genes, are needed before definitively concluding that differential pleiotropy explains the highly biased involvement of the *Myb* gene in evolutionary transitions to white or yellow flowers.

One caveat to this argument is that cis-regulatory mutations that reduce the expression of an enzyme-coding gene or regulatory gene only in flowers would also be expected to incur minimal pleiotropy, yet these have not been seen in evolutionary transitions. In fact, such cis-regulatory mutations that inactivate only the promoter module responsible for expression in flowers likely have a very small ‘target size’ and are rare. Of 69 spontaneous mutations reducing floral pigmentation tabulated by Streisfeld and Rausher (2010), only eight were cis-regulatory. Of these, most involved either transposon insertions or large deletions in the promoter region, which likely inactivate the gene in all tissues. Although technically these are cis-regulatory mutations, they would experience the same magnitude of deleterious pleiotropy as knockouts of enzyme-coding genes.

Evolutionary transitions from blue to red flowers

A second common evolutionary transition is from blue or purple to red flowers (Fig. 1). This type of shift often accompanies shifts in adaptation from bee to hummingbird, co-occurs with other floral adaptations that facilitate hummingbird pollination, and can arise repeatedly within a plant genus or family (Rausher, 2008; Thomson and Wilson, 2008). Shifts from bluer to redder flowers generally involve a change in the biochemical class of anthocyanin that is produced. Each type of flavonoid, including anthocyanins, can occur in three different forms that vary in the number of hydroxyl groups attached to the B-ring of the molecule. Anthocyanins derived from pelargonidin have one hydroxyl group, those derived from cyanidin have two, and those derived from delphinidin have three. In general, an increase in hydroxyl groups shifts the hue of the molecule from redder to bluer: pelargonidin-based anthocyanins tend to be red or orange, cyanidin-based anthocyanins range from reddish-magenta to
blue/purple (depending on the presence of co-pigments), and delphinidin-based anthocyanins tend to be blue/purple. Not surprisingly, in both spontaneous mutations and evolutionary substitutions, shifts from bluer to redder flowers almost always involve a decrease in hydroxylation state, although there are much less common types of mutations that involve elimination of co-pigments (Scott-Moncrieff, 1936; Takahashi et al., 2006).

The production of cyanidin and other dihydroxylated flavonoids requires the activity of flavonoid 3’-hydroxylase (F3’H). The production of delphinidin and other trihydroxylated flavonoids requires the activity of flavonoid 3’,5’-hydroxylase (F3’5’H). Shifts from more- to less-hydroxylated anthocyanins typically involve the inactivation of one or more of these hydroxylating enzymes, which redirect flux through the appropriate branch of the pathway. Inactivation of F3’5’H results in a shift from delphinidin to cyanidin if F3’H is also expressed or a shift from delphinidin to pelargonidin if not. Inactivation of F3’H can cause a shift from cyanidin to pelargonidin in the absence of F3’5’H expression. Inactivation of either gene could be accomplished by: (1) a functional mutation to the enzyme; (2) a cis-regulatory mutation that downregulates the enzyme; or (3) a functional mutation to an anthocyanin transcription factor that specifically affects the expression of the enzyme. Again, we consider mutations in category 1 to be functional and those in categories 2 and 3 to be regulatory.

Although the sample size is still small, the predominance of regulatory vs. functional mutations involved in the evolution of red flowers displays intriguing patterns. Spontaneous mutations causing red flowers occur through LOF mutations in coding sequences of both F3’H and F3’5’H, with no cases of spontaneous regulatory mutations (Supplementary Table S1). The exception is Ipomoea ternifolia, in which a cis-regulatory mutation downregulates F3’5’H, although the functionality of this gene in red-flowered individuals has not been tested (Hopkins and Rausher, 2011).

Mutations to F3’H that contribute to evolutionary divergence in flower colour do not reflect the spontaneous mutation rate – despite the higher rate of functional mutations, only regulatory mutations with effects restricted to floral tissue have been fixed by natural selection. On the other hand, mutations to F3’5’H involved in flower colour divergence do largely reflect the spontaneous mutation rate – most involve functional inactivation. These patterns suggest that functional mutations to F3’H, but not F3’5’H, have low fitness relative to regulatory mutations due to associated pleiotropic effects. The major pleiotropic roles of F3’H and F3’5’H are the production of di- and trihydroxylated flavonoids in vegetative tissues, respectively. We have compiled several lines of evidence that the production of dihydroxylated flavonoids, but not trihydroxylated flavonoids, in vegetative tissue may be important for plant fitness. This evidence is consistent with the interpretation that LOF mutations in F3’H, but not F3’5’H, incur substantial deleterious pleiotropy.

First, we compared the relative abundance of the different anthocyanins found in flowers vs. vegetative tissue reported by biochemical surveys of anthocyanin production (Table 2). Floral anthocyanidin production is quite variable among species surveyed: 20% produce pelargonidin, 44% produce cyanidin, and 45% produce delphinidin. Despite this variability in floral tissue, most plant species (nearly 90%) produce only the dihydroxylated cyanidin in vegetative tissue. This pattern suggests that most species that produce pelargonidin or delphinidin in flowers produce cyanidin in their foliage, consistent with natural selection favouring production of dihydroxylated flavonoids in vegetative tissue.

Second, differences in the taxonomic distributions of F3’H and F3’5’H also suggest that a loss of F3’H activity is substantially more costly than a loss of F3’5’H activity. The incorporation of both F3’H and F3’5’H into the anthocyanin pathway occurred

<table>
<thead>
<tr>
<th>Blue species</th>
<th>Red species</th>
<th>Gene action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ipomoea ternifolia: cy</td>
<td>Ipomoea coccinea: pg</td>
<td>F3’H – regulatory</td>
<td>Des Marais and Rausher, 2010</td>
</tr>
<tr>
<td>Ipomoea purpurea: cy</td>
<td>Ipomoea horsemalliae: pg</td>
<td>F3’H – regulatory</td>
<td>Des Marais and Rausher, 2010</td>
</tr>
<tr>
<td>Ipomoea purpurea: cy</td>
<td>Ipomoea quamoclit: pg</td>
<td>F3’H – regulatory</td>
<td>Des Marais and Rausher, 2010</td>
</tr>
<tr>
<td>Iochroma cyaneum: de</td>
<td>Iochroma gesnerioides: pg</td>
<td>F3’H – regulatory</td>
<td>Zufall and Rausher, 2004; Des Marais and Rausher, 2010</td>
</tr>
<tr>
<td>Iochroma cyaneum: de</td>
<td>Iochroma gesnerioides: pg</td>
<td>F3’H – coding</td>
<td>Smith and Rausher, 2011</td>
</tr>
<tr>
<td>Penstemon neomexicanus: de</td>
<td>Penstemon barbatus: pg</td>
<td>F3’5’H – coding (and regulatory)</td>
<td>Unpublished data</td>
</tr>
<tr>
<td>Antirrhinum kelloggii: de</td>
<td>Antirrhinum majus: cy</td>
<td>F3’5’H – coding</td>
<td>Ishiguro et al., 2011</td>
</tr>
<tr>
<td>Phlox drummondii (purple subsp.): de</td>
<td>Phlox drummondii (pink subsp.): cy</td>
<td>F3’5’H – regulatory</td>
<td>Hopkins and Rausher, 2011</td>
</tr>
</tbody>
</table>

Comparisons are between anthocyanins produced in flowers of blue-flowered species and a closely related red-flowered species. Gene action denotes whether the mutation is a regulatory mutation causing downregulation of indicated gene, or a loss-of-function (functional) mutation. cy, cyanidin; de, delphinidin; pg, pelargonidin.
prior to the beginning radiation of angiosperms (Rausher, 2006; Seitz et al., 2006), implying that absence of either gene from any flowering plant is due to gene loss. We are unaware of any plants that lack a functional copy of $F3'h$; if they exist, they are almost certainly rare. $F3'5'h$, however, appears to have been completely lost in a number of taxa including Rosaceae, most of the Asteraceae, Antirrhinum majus, Arabidopsis thaliana, Ipomoea, Iochroma gesnerioides, Matthiola, and Tulipa. Note that this list represents a large proportion of taxa where the presence of $F3'5'h$ has been explicitly investigated and likely is the tip of the iceberg for taxa lacking $F3'5'h$. If one maps these absences onto the most recent consensus tree for angiosperms (Stevens, 2001, version 9) along with taxa that are known to have a functional $F3'5'h$ because they produce trihydroxylated flavonoids, it is clear that there have been numerous independent losses (Supplementary Fig. S1). This difference between $F3'5'h$ and $F3'h$ in rate at which LOF mutations accumulate is most easily interpreted as reflecting more stringent constraint, due to greater deleterious pleiotropy, of LOF mutations in $F3'h$.

Finally, there is substantial evidence that dihydroxylated flavonoids perform various physiological functions better than the corresponding mono- or trihydroxylated flavonoids. Flavonols are among the most abundant group of flavonoids (Harborne, 1967) and carry out a variety of functions in plants (reviewed by Winkel-Shirley, 2002; Taylor and Grotewold, 2005; Pollastri and Tattini, 2011). One important role is protection against the harmful effects of environmental stress. Under certain stressful conditions, plants accumulate flavonols, in particular the dihydroxylated flavonol quercetin, thus dramatically increasing the total flavonol content and also the ratio of quercetin to the monohydroxylated flavonol kaempferol in tissues exposed to the stress (reviewed by Pollastri and Tattini, 2011). This has most commonly been observed in response to high light and UVB radiation (e.g. Olsson et al., 1998; Ryan et al., 1998, 2001, 2002; Gerhardt et al., 2008; Agati et al., 2009, 2011), but has also been documented in response to salinity stress (Agati et al., 2011), drought stress (Tattini et al., 2004), and nitrogen depletion (reviewed by Lillo et al., 2008). This physiological response appears to be an adaptive mechanism to reduce the harmful effects of reactive oxygen species produced by these environmental stresses. Flavonols have important antioxidant properties conferred by their ability to chelate transition metal ions (Rice-Evans et al., 1996). Importantly, the dihydroxylated quercetin is significantly better at chelating both Cu ions (Brown et al., 1998; Mira et al., 2002) and Fe ions (Mira et al., 2002; Melidou et al., 2005) than the monohydroxylated kaempferol. This provides a convincing explanation for why quercetin in particular is induced by environmental stresses, and it has been suggested that this protective function is so essential that it has generated strong purifying selection on mutations reducing the ability to produce quercetin throughout higher plants (Pollastri and Tattini, 2011).

Flavonols also act as developmental modulators at multiple positions within the auxin hormone signalling pathway, enabling them to influence plant architecture, root morphology, gravitropism, and other phenotypes sensitive to auxin (reviewed by Taylor and Grotewold, 2005). Both quercetin and kaempferol can bind to auxin transporters and inhibit polar auxin transport (Jacobs and Rubery, 1988; Murphy et al., 2000; Brown et al., 2001). Data provided by Jacobs and Rubery (1988) suggest that quercetin has a stronger affinity for auxin transporter binding sites compared to kaempferol, a result that has been frequently cited in subsequent papers, although Jacobs and Rubery did not report a statistical comparison. If there were a statistically significant difference, it would suggest that quercetin is a more important inhibitor of polar auxin transport than kaempferol. In support of this conclusion, Arabidopsis thaliana mutants that lack $F3'h$ activity (and therefore can produce kaempferol, but not quercetin) have similar auxin transport phenotypes as mutants that completely lack flavonols (Lewis et al., 2011). This result suggests that normal auxin transport depends upon the ability to produce quercetin. Furthermore, quercetin and kaempferol have different effects on auxin degradation. Quercetin inhibits peroxidase-mediated degradation of endogenous auxin (IAA), while kaempferol acts as a cofactor in this pathway (Furuya et al., 1962). Interestingly, UV radiation induces the expression of the peroxidases that degrade IAA (Jansen et al., 2001) in addition to increasing the ratio of quercetin to kaempferol, as discussed above. UV radiation has known impacts on auxin metabolism, although it is unclear whether this occurs as a direct response to UV radiation or an indirect response mediated through UV-responsive flavonols (reviewed by Jansen, 2002). Combined with the observation that auxin itself stimulates the flavonoid pathway genes, including $F3'h$ (Lewis et al., 2011), it seems possible that there is a complicated regulatory pathway that integrates signals from UV radiation and auxin metabolism which possibly relies upon the presence of quercetin.

Finally, it is worth noting that a variety of flavonoid compounds also have important roles as antimicrobial and antiherbivory compounds (reviewed by Harborne and Williams, 2000). Proanthocyanidins are particularly important antiherbivory compounds (reviewed by Dixon et al., 2004). The majority of proanthocyanidins found in nature are dihydroxylated (procyanidin), while propelargonidin and prodelphinidin are rare (Tanner, 2004), suggesting procyanidins are more effective, although we have not found direct documentation of this.

These various lines of evidence are all consistent with the hypothesis that functional inactivation of $F3'h$ is very costly, whereas inactivation of $F3'5'h$ is not. If this hypothesis is correct, it would explain why shifts to red flowers are associated with downregulation of $F3'h$ but functional inactivation of $F3'5'h$. Downregulation of $F3'h$ only in floral tissue avoids the expected deleterious pleiotropic effects that would be associated

### Table 2. Proportions of species containing major classes of anthocyanins in floral and vegetative tissue

<table>
<thead>
<tr>
<th>Anthocyanin class</th>
<th>Floral tissue $(n = 860)$</th>
<th>Vegetative tissue $(n = 376)$</th>
</tr>
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<tbody>
<tr>
<td>Derived from pelargonidin</td>
<td>0.199</td>
<td>0.016</td>
</tr>
<tr>
<td>Derived from cyanidin</td>
<td>0.442</td>
<td>0.891</td>
</tr>
<tr>
<td>Derived from delphinidin</td>
<td>0.448</td>
<td>0.160</td>
</tr>
</tbody>
</table>

with functional inactivation of F3’h. Functional inactivation of F3’5’h, on the other hand, would have no effect on the production of dihydroxylated flavonoids in vegetative tissues and would not be expected to experience greater pleiotropic costs than mutants that downregulate F3’5’h. Since functional mutations arise at a higher rate, they would predominantly underlie most evolutionary transitions from delphinidin- to cyanidin-based floral anthocyanins. While the observed pattern is consistent with this explanation, more direct evidence on the magnitude of deleterious pleiotropy associated with mutations to these two genes is needed.

Lessons from the genetics of flower colour evolution

Several lessons flow from patterns revealed by data on the genetic basis of flower colour adaptation. The first is that broad generalizations, such as ‘morphological traits tend to involve regulatory change’ or ‘physiological traits tend to involve functional coding-sequence change’ fail to predict differences among very similar traits. Regardless of whether flower colour is considered a morphological or a physiological trait, adaptation can involve primarily regulatory or primarily functional genetic changes, depending on the type of flower colour change and the pathway position of the genes involved. Transitions to white or yellow flowers involve regulatory changes mediated by a specific transcription factor (R2R3MYB). Transitions from blue to red flowers primarily involve functional mutations if the ancestral blue flowers are coloured by delphinidin, but involve primarily cis-regulatory mutations if the ancestral blue flowers are coloured by cyanidin. These differences indicate that flower colour shifts, even phenotypically identical shifts, may predictably involve different types of genetic mutations.

The second lesson is that differences in pleiotropy often determine whether regulatory vs. functional mutations are preferentially involved in adaptive evolution. Minimal deleterious pleiotropy appears to explain why virtually all cases of transition to unpigmented flowers involve inactivation of the R2R3MYB anthocyanin transcription factor. It also explains why shifts to red flowers preferentially involve downregulation of F3’h. In the case of F3’5’h, there is likely little difference among regulatory and functional mutations in pleiotropy, and the prevalence of functional mutations appears to be determined largely by mutational target size.

A third lesson is that determining whether we can or cannot predict the genetic basis of an adaptive trait is facilitated by examining the genetics of its repeated evolutionary origins (see also Kopp, 2009). If enough cases are investigated, this approach allows a powerful statistical assessment of whether different types of mutation are preferentially involved in different types of evolutionary transitions and in different traits. In the case of floral pigmentation loss, a sufficient number of cases have been examined to produce a statistically convincing case that R2R3Myb mutations are preferentially fixed. The case for the patterns associated with blue/purple to red transitions is less secure because it rests on fewer cases. However, additional cases are being examined, and it should not be too long before the statistical confidence for this transition is as high as for loss of pigments.

A final lesson is that evolutionary novelty can be produced by loss of function at the molecular level. While it is unclear whether floral pigmentation is an ancestral trait in angiosperms, it is clearly an ancestral trait of many angiosperm families that exhibit evolutionary changes in flower colour, as is blue/purple floral pigmentation. Evolutionary transitions to white or red flowers thus constitute novel phenotypes. Nevertheless, the mutations underlying these transitions are overwhelmingly loss-of-function mutations that either inactivate protein function or greatly reduce protein expression in floral tissues. We know of only one gain-of-function mutation responsible evolutionary change in flower colour: a cis-regulatory mutation in the floral anthocyanin R2R3Myb gene in Phlox drummondii upregulates that gene’s expression level and causes an increase in anthocyanin pigment production (Hopkins and Rausher, 2011). It is perhaps not surprising that the magnitude of deleterious pleiotropy will play a large role in determining which LOF mutations are preferentially involved in the evolution of flower colour. In principle, we would expect the magnitude of deleterious pleiotropy to also influence which gain-of-function mutations contribute disproportionately to evolution of plant traits, since the magnitude of pleiotropy will affect the net selection coefficients of those mutations. Only future investigations can determine whether this is the case. We suggest that a profitable approach for such investigations is the genetic dissection of repeated evolutionary transitions to the same novel phenotype.

Supplementary material

Supplementary data are available at JXB online.

Supplementary Table S1. Cases identified from the literature in which a spontaneous mutation to flower colour causes a shift from more- to less-hydroxylated anthocyanins.

Supplementary Fig. S1. Angiosperm phylogenetic tree showing loss or functional inactivation of F3’5’h.

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