

Genetic changes contributing to the parallel evolution of red floral pigmentation among *Ipomoea* species

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Summary

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- The repeated, independent evolution of phenotypic traits reflects adaptation to similar selective pressures. In some circumstances, parallel phenotypic evolution has a common genetic basis. Here, we investigate the types of genetic change responsible for the repeated evolution of red flowers among *Ipomoea* species.
- We identified three independent transitions from cyanidin- (blue/purple) to pelargonidin-type (red) anthocyanin pigments among *Ipomoea* species. The genetic basis for these transitions was examined using transgenics and gene expression assays. Using a literature survey to estimate the expected spectrum of mutation types capable of producing red flowers, we evaluated whether the observed distribution of mutation types differed from expectation.
- In these species, red floral pigmentation appears to be caused by the disruption of flux through the anthocyanin pathway at the same position. Results implicate tissue-specific regulatory changes in the same gene, which suggests the possibility that flower color evolved independently via the same genetic mechanism.
- Although multiple molecular mechanisms are capable of producing red flowers, we found a deviation between the distributions of observed and expected mutation types responsible for these evolutionary transitions. Regulatory mutations thus appear to be preferentially targeted during evolutionary change between species. We discuss possible explanations for this apparent bias.

Abbreviations: ANS, anthocyanidin synthase; CaMV, cauliflower mosaic virus; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol-4-reductase; F3H, flavonoid-3-hydroxylase; F3'H, flavonoid-3'-hydroxylase; F3'5'H, flavonoid-3',5'-hydroxylase; FLS, flavonol synthase; RACE, rapid amplification of cDNA ends; RT-PCR, reverse-transcribed polymerase chain reaction; *tt*, *transparent testa*; UF3GT, UDP-flavonoid-3-glucosyl-transferase.

Introduction

The repeated, independent evolution of similar characters from a common ancestral state, called parallel evolution, often reflects a signature of adaptation caused by similar environmental selective pressures. Recently, evolutionary biologists have begun to characterize, at the molecular level, the genetic basis for this phenomenon. In a variety of systems, it has been found that changes in the same gene, sometimes involving the same substitution, are responsible for the independent origins of the same character, although other systems do not exhibit this

pattern (reviewed in Arendt & Reznick, 2008). At least three possible explanations can account for parallel evolution at the molecular level: the observed mutation is the only one possible for producing the novel phenotypic character; this mutation arises more often than the others; or certain mutations are fixed preferentially by natural selection, whereas others are not, presumably because of differences in constraint on mutations in alternative genes.

In order to distinguish among these possibilities, we need to compare examples of the genetic basis of parallelism from multiple, independent evolutionary transitions with an unbiased estimate that reflects the total spectrum of mutations capable of producing the novel phenotype. One way to obtain such an estimate is to use information on genetically characterized

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos GQ180934–GQ180936.

spontaneous mutations that arise in horticultural accessions or that segregate in natural populations. These mutations have arisen recently and have not been eliminated by natural selection, suggesting that they represent a rough approximation of the overall distribution of mutations capable of producing this phenotype. For example, if there is evidence for multiple types of mutations generating the phenotype, we can rule out the first possibility that only one mutation is capable of producing the phenotype. Alternatively, if the same mutation is implicated and over-represented in both the evolutionary transitions and spontaneously mutated samples, it suggests that this mutation arises more commonly than other mutations that can produce the same phenotype. Finally, if the observed frequency of mutations responsible for the evolutionary transitions differs from the distribution of spontaneous mutation types, we can infer that the observed parallelism probably is caused by the preferential fixation of these mutations by selection. However, for most characters that exhibit parallel phenotypic and molecular evolution, rarely do we know the frequencies of spontaneous mutations that can generate a particular phenotype. We thus have little information on the molecular processes that explain genetic parallelism.

Flower color is an ideal trait for examining these issues. Evolutionary transitions in flower color are numerous (Rausher, 2008), and many of these transitions involve changes in either the type or quantity of anthocyanin pigments that are produced. Moreover, the anthocyanin biosynthetic pathway is highly conserved across angiosperms (Holton & Cornish, 1995). As a consequence of the extensive investigation into the function and regulation of the genes comprising this pathway, the genes encoding the pathway enzymes and the major transcription factors that regulate their expression have been characterized for a variety of model species (Grotewold, 2006a). This previous work facilitates the identification of the genetic changes responsible for floral color evolution in nonmodel systems (for example, Streisfeld & Rausher, 2009). Finally, information on spontaneous mutations in horticultural lines or on variants segregating within populations provides information that allows one to compare the genes used in evolutionary transitions of flower color with those involved in spontaneous mutations yielding the same shift in color.

Accumulating evidence indicates that flower color transitions involving pigmented flowers and flowers lacking anthocyanin pigments (white or yellow flowers) often are a result of mutations in transcription factors (four of four cases examined; Quattrocchio *et al.*, 1999; Schwinn *et al.*, 2006; Cooley, 2008; Streisfeld & Rausher, 2009). Two additional investigations provide evidence consistent with the involvement of transcription factors, but are not definitive because they fail to rule out nonfunctionality of anthocyanin pathway enzymes (Durbin *et al.*, 2003; Whittall *et al.*, 2006). To date, however, the frequency with which mutations in transcription factors occur in nature relative to spontaneous mutations in other anthocyanin genes has not been examined, and so it is unclear whether

transcription factors are preferentially targeted during these evolutionary transitions.

A second, common type of flower color transition that occurs repeatedly in some plant groups is a shift from blue or purple, typically insect-pollinated flowers, to red, hummingbird-pollinated flowers (Kay *et al.*, 2005; Wilson *et al.*, 2007; Rausher, 2008). These transitions frequently are achieved by altering the flux down different branches of the anthocyanin pathway (Fig. 1), and they often involve changes from derivatives of the cyanidin or delphinidin pigments to the red, pelargonidin pigment (for example, Scogin & Freeman, 1987; Zufall & Rausher, 2004; Rausher, 2008). Other alterations, such as changes in vacuolar pH (Fukada-Tanaka *et al.*, 2000; Quattrocchio *et al.*, 2006b), epidermal cell shape (Noda *et al.*, 1994) or in the decorative moieties attached to anthocyanins (Nakajima *et al.*, 2005) can also cause floral hue modification, but they appear to be less common than changes in pigment type. Despite the commonness of this type of floral color transition, the genetic changes responsible have been documented for only one species, *Ipomoea quamoclit* (Des Marais, 2008). Consequently, whether certain types of genetic change are used repeatedly in these transitions is unknown.

In this study, we begin to address these issues by characterizing the nature of changes responsible for shifts from blue to red flowers in two additional species, *Ipomoea udeana* and *Ipomoea horsfalliae*. We first perform a phylogenetic analysis of *Ipomoea* species to demonstrate that the presence of red flowers among these species represents independent evolutionary transitions in this character. We then address two questions. Are these transitions caused by similar mutations among species? Can we gain insight from information on spontaneously occurring and segregating natural mutants about which molecular processes are responsible for the parallel genetic changes causing these evolutionary transitions?

Materials and Methods

Study system

In the morning glories (*Ipomoea* L., Convolvulaceae), primarily bee-pollinated, blue/purple flowers producing cyanidin are believed to constitute the ancestral state for flower color (McDonald, 1991). Previous work has identified three independent origins of red flowers in *Ipomoea* (Zufall, 2003). For two of these transitions (members of the *Mina* clade of subgenus *Quamoclit*, and *I. udeana*), only pelargonidin pigments are produced in the flowers. *Ipomoea quamoclit* produces cyanidin derivatives in vegetative tissue (Des Marais, 2008), but the type of pigments produced in vegetative tissues of *I. udeana* is unknown. Initial work on one species in the *Mina* clade (*I. quamoclit*) suggested that the transition from cyanidin to pelargonidin production in flowers could be attributed to multiple changes that affected the expression or function of two anthocyanin pathway enzymes (Zufall & Rausher, 2004).

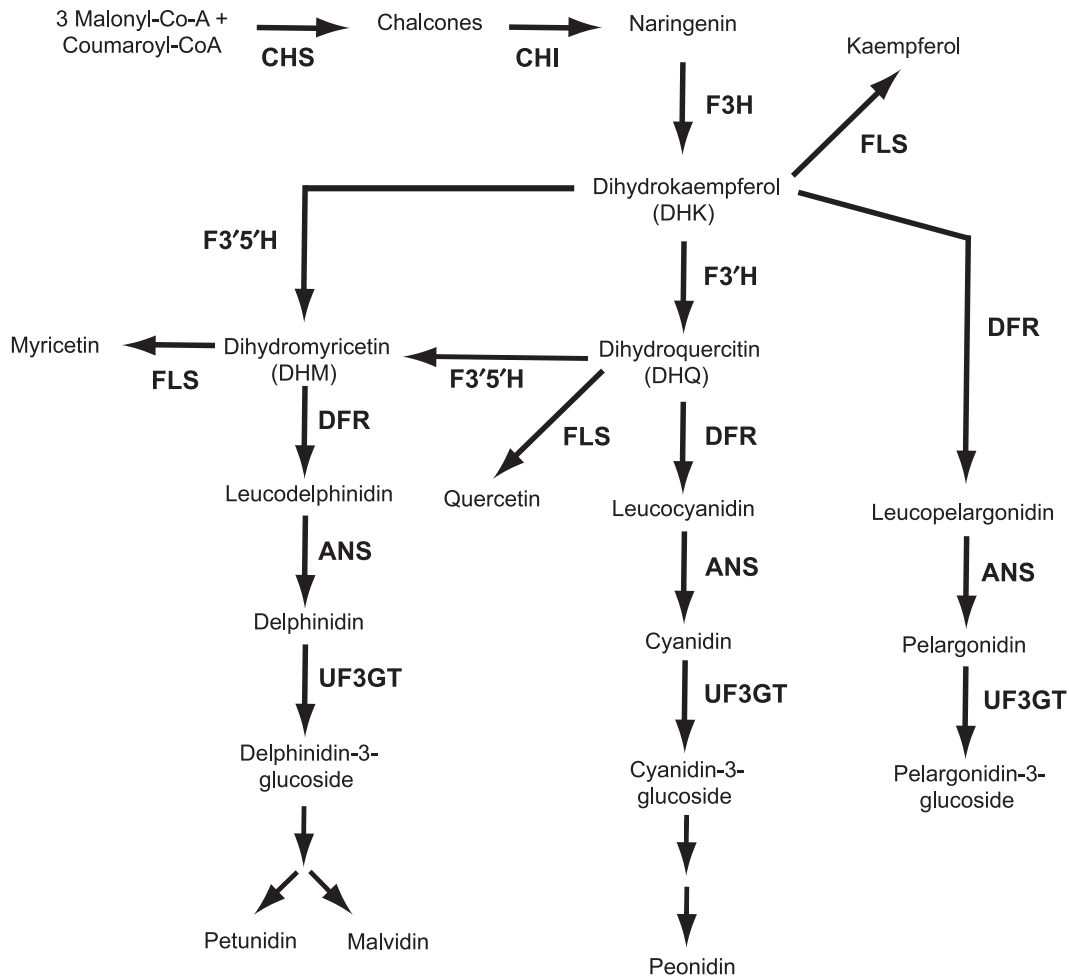


Fig. 1 A schematic diagram of the three major branches of the anthocyanin biosynthetic pathway. Each enzyme is listed as an abbreviation beside the arrows, and the products of each enzymatic reaction are listed below the arrows. The FLS enzyme is not required for pigment production, but is necessary for the synthesis of the three flavonols: kaempferol, quercetin and myricetin. Modified forms of the anthocyanidins are shown below the cyanidin and delphinidin branches. Abbreviations: ANS, anthocyanidin synthase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol-4-reductase; F3H, flavonoid-3-hydroxylase; F3'H, flavonoid-3'-hydroxylase; F3'5'H, flavonoid-3',5'-hydroxylase; FLS, flavonol synthase; UF3GT, UDP-flavonoid-3-glucosyl-transferase.

More recent investigations have shown convincingly that only change to the *cis*-regulatory region of the flavonoid-3'-hydroxylase gene (*F3'H*) causes a tissue-specific reduction in gene expression that appears to be necessary and sufficient for the transition to pelargonidin production in flowers (Des Marais, 2008). By contrast, *I. conzattii* makes red flowers, but does so with cyanidin pigments, demonstrating that there are multiple genetic mechanisms for obtaining a similar red color phenotype (Zufall, 2003).

For this study, we examine an additional red-flowered species, *Ipomoea horsfalliae*, which has also been demonstrated to produce exclusively pelargonidin in its flowers (Forsyth & Simmonds, 1954). In addition, we analyze pigment production in the vegetative tissues of *I. udeana*. Because floral tissue was unavailable, we did not conduct genetic analyses on this species.

Phylogenetic reconstructions

The purpose of our phylogenetic reconstruction was to determine whether the evolution of red flowers in *I. horsfalliae* represented an event evolutionarily independent of the same transitions in *I. quamoclit* and *I. udeana*. The monophyletic tribe Ipomoeae (Convolvulaceae) consists of two well-supported clades, the Astripomoeinae and the Argyreinae, which contains the previously described genera *Ipomoea*, *Stictocardia*, *Astripomoea*, *Calonyction* and *Turbina* (Stefanovic *et al.*, 2003). We reconstructed the phylogeny of the Astripomoeinae using internal transcribed spacer (ITS) sequences from 30 previously analyzed taxa (Miller *et al.*, 1999, 2004; ITS sequences from *I. horsfalliae* and *I. udeana* kindly provided by R. Miller). We employed Bayesian estimation in MrBayes v. 3.1 (Ronquist & Huelsenbeck, 2003) using the GTR + GI models, which

consisted of two runs with five million generations each of four Markov chain Monte Carlo (MCMC) chains to generate a posterior distribution containing 15 000 trees following burn in. The substitution model was selected according to hierarchical likelihood ratio tests, as implemented in MrModeltest v. 2.2 (Nylander, 2004). Parameter convergence was evaluated according to Ronquist & Huelsenbeck (2003). We used two methods to estimate the mean and variance of the number of transitions to and from red flowers in this group. First, we used parsimony to trace the history of the unordered binary floral colors (red or not red) across 200 trees randomly sampled from the Bayesian posterior distribution of trees, as implemented in Mesquite v. 2.5 (Maddison & Maddison, 2008). Next, we simulated the character histories across 300 realizations of each of 200 randomly selected trees using stochastic mapping, as implemented in SIMMAP v. 1.0 (Bolback, 2006). This program takes into account phylogenetic uncertainty by integrating across the posterior distribution of trees, and explicitly allows for multiple transitions along individual branches. We calculated the mean and standard deviation of the number of transitions to and from red flowers across all of the simulated histories.

Anthocyanin pathway and genes examined

At least six different enzymatic reactions must occur to produce pigments, and the genes coding for these enzymes have been characterized in several model species (Fig. 1; Holton & Cornish, 1995). With a few exceptions (Grotewold, 2006b), all anthocyanin pigments are derived from one of three anthocyanidin precursors: cyanidin, pelargonidin and delphinidin. These precursors are produced by alternative branches of the pathway (Fig. 1). The production of cyanidin and its derivatives requires the expression and correct function of the branching enzyme F3'H, whereas the production of delphinidin and its derivatives also requires correct expression and function of the branching enzyme flavonoid-3',5'-hydroxylase (F3'5'H). In *Ipomoea*, the delphinidin branch is absent, and pigments derived from cyanidin are typically blue–purple, whereas those derived from pelargonidin are typically red (Rauscher, 2006).

In principle, flux down the cyanidin branch of the pathway can be blocked and redirected down the pelargonidin branch by modifying one of three genes. First, pelargonidin could be produced by two types of mutation affecting the gene encoding F3'H: either mutations that eliminate the expression of the gene (for example, Des Marais, 2008) or mutations in the coding sequence that render the enzyme nonfunctional (for example, Hoshino *et al.*, 2003; Zufall & Rauscher, 2003). Second, it is predicted that a transition to pelargonidins could be achieved by alteration of the substrate specificity of dihydroflavonol-4-reductase (DFR). In blue-flowered *Ipomoea* species that have been examined, DFR is a substrate generalist, meaning that it can metabolize precursors of both pelargonidin and cyanidin (Des Marais & Rauscher, 2008). A mutation that prevents DFR from metabolizing the cyanidin precursor would block

the cyanidin branch of the pathway and presumably redirect flux down the pelargonidin branch. DFR enzymes with substrate specificity are known from several plant species (Johnson *et al.*, 2001; Rauscher, 2006). Although, in these examples, the ability to metabolize the pelargonidin precursor has been lost, in principle, there seems to be no reason to believe that the ability to metabolize the cyanidin precursor could not be lost instead. Finally, although we are unaware of any examples, it also is conceivable that substrate specificity could evolve in the enzyme anthocyanidin synthase (ANS). We thus focus our analyses on F3'H, DFR and ANS as possible candidates for the transition from cyanidin to pelargonidin in the flowers of *I. horsfalliae*.

Pigment extractions

Previous work on *I. quamoclit* has shown that anthocyanins produced in foliage are derived from cyanidin, demonstrating that different branches of the pathway are active in flowers and vegetative tissues of the same plant (Des Marais, 2008). In order to determine the type of pigment produced in the vegetative tissues of *I. horsfalliae* and *I. udeana*, we extracted anthocyanidins from 500 mg of stem tissue according to previously described methods (Harborne, 1984; Streisfeld & Rauscher, 2009). Forty microliters of the resuspended extract were injected into a Shimadzu LC-10AT liquid chromatograph (Kyoto, Japan) with a 4.6 × 150 mm Alltech Prevail reverse phase C18 column (Alltech Associates, Deerfield, IL, USA) at a flow rate of 1 ml min⁻¹. Separation and detection were carried out as described previously (Streisfeld & Rauscher, 2009). Retention times were compared with a mixture of six commercially prepared standards (cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin; Poyphenols Laboratories, Sandnes, Norway) run under similar conditions.

Cloning of candidate genes

In order to obtain full-length coding sequences of the three candidate genes from *I. horsfalliae*, we first used reverse-transcribed polymerase chain reaction (RT-PCR) and primers designed from conserved regions based on multiple, previously published sequences from *Ipomoea* to obtain partial coding sequences of these genes. Total RNA was extracted from the distal half of flower buds 1 d before anthesis using a Qiagen RNeasy kit (Qiagen, Valencia, CA, USA). First-strand cDNA synthesis was performed from 500 ng of RNA using M-MLV reverse transcriptase and the Gene-Racer oligo-dT primer, according to the manufacturer's specifications (Invitrogen, Carlsbad, CA, USA). All products were gel extracted (Qiagen) and cloned into the pCR 2.1 TOPO vector from Invitrogen, and plasmids were sequenced with the M13 forward and reverse primers. BLAST searches against the National Center for Biotechnology Information (NCBI) nucleotide and protein databases were used to confirm homology with previously characterized anthocyanin genes. We next used 5' and 3' rapid

amplification of cDNA ends (RACE) procedures to obtain the remaining portions of the coding region from these genes. Finally, PCR from cDNA was performed to obtain full-length coding sequences of these genes using high-fidelity PFX polymerase (Invitrogen). These full-length products were cloned as above into the pCR 2.1 TOPO vector and the plasmids were sequenced to confirm the presence of the inserts. Although it is known that *Dfr* is a member of a three-gene family in *Ipomoea* (Inagaki *et al.*, 1999), only the *Dfr-B* copy retains activity on the dihydroflavonols in *Ipomoea* (Des Marais & Rausher, 2008). To ensure that we cloned this copy, we compared our sequence with previously identified orthologs from *Ipomoea* (Des Marais & Rausher, 2008). In order to verify that we identified single copies of *F3'H* and *Ans*, we compared full-length sequences of each gene obtained from PCR of floral bud cDNA with that obtained from stem cDNA.

Full-length products were released from pCR 2.1 by *EcoRI* digestion and subcloned into the *EcoRI* site of the pCB vector. This step allowed *BamHI* and *XhoI* sites to be added to the ends of the inserts for subsequent cloning into the binary vector pBI 1.4. *BamHI/XhoI* fragments from pCB were cloned into pBI 1.4, putting the anthocyanin genes under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter. This process was completed for each of the three candidate genes, yielding constructs pB-*Ih_F3'H*, pB-*Ih_DFR*, and pB-*Ih_ANS*. pBI 1.4 contains the plant selectable *nptII* gene for kanamycin resistance. All clones were sequenced at every step to guarantee that no errors were introduced into the plasmids and to confirm the correct orientation of the insert.

Transgenic complementation tests

To test whether the three candidate genes from *I. horsfalliae* produced functional enzymes *in vivo*, we used a previously described transgenic complementation approach that takes advantage of the individual *transparent testa* (*tt*) mutants from *Arabidopsis thaliana* that are deficient in anthocyanin production in seedlings (Zufall & Rausher, 2003, 2004). In *Arabidopsis*, it is known that each of the core anthocyanin enzymes is encoded by a single-copy gene. By using *Arabidopsis* lines lacking function of the different anthocyanin enzymes, we could assay whether the introduction of a construct containing an over-expressed gene from *I. horsfalliae* could restore anthocyanin production in *Arabidopsis* seedlings, and thus complement the loss-of-function mutation. Moreover, because the pelargonidin branch of the anthocyanin pathway is inactive in *A. thaliana*, wild-type plants produce only cyanidin-derived anthocyanins (Dong *et al.*, 2001). Consequently, restoration of anthocyanin production by the transgene in these assays indicates the ability of *I. horsfalliae* DFR and ANS to metabolize cyanidin precursors. For each of the three candidate genes, we obtained the *tt* mutants deficient for the enzymatic function encoded by that gene from the Arabidopsis Biological Resource

Center (*tt7*, lacking F3'H activity; *tt3*, lacking DFR activity; and *tt18* lacking ANS activity).

The pB-*Ih_F3'H*, pB-*Ih_DFR* and pB-*Ih_ANS* constructs were transformed into *Agrobacterium tumefaciens* strain GV3101. Each construct was introduced into the corresponding *tt* mutant line using the standard floral dip procedure (Clough & Bent, 1998). Plants were grown in standard growth chambers under a 16 h : 8 h light : dark regime at 22°C. Following dipping, plants were allowed to flower and seeds were harvested. Positive transformants among the offspring (T1 generation) were selected on agar plates containing Murashige and Skoog salts, supplemented with 2% (w/v) sucrose and 50 µg ml⁻¹ of kanamycin, followed by transplantation to soil and allowing them to set seed. The offspring (T2 generation) were then tested for their ability to complement the loss-of-function mutations by germinating and growing seedlings on sterile sand under high light. These growth conditions provide sufficient nutrient and light stress for the induction of anthocyanin production in the cotyledons of wild-type plants within 7–10 d following germination (Zufall & Rausher, 2004; Lillo *et al.*, 2008). Four lines transformed with each construct were tested for complementation by visualizing the production of the purple anthocyanins in the cotyledons 10 d after germination. For each positively transformed line, we extracted genomic DNA from leaf tissue and performed PCR to confirm the presence of the transgene using primers specific to a portion of the coding sequence from *I. horsfalliae*.

Gene expression

We assayed the relative expression levels of the three candidate genes from floral and stem tissue in *I. horsfalliae* using quantitative RT-PCR (qPCR). We extracted total RNA from the distal half of floral buds 1 d before anthesis. This developmental stage has been shown to be appropriate for the identification of anthocyanin gene transcripts from several species of *Ipomoea* (for example, Tiffin *et al.*, 1998; Zufall & Rausher, 2004; Morita *et al.*, 2006). In addition, RNA was extracted from the outer layer of the stem from *I. horsfalliae* on first appearance of pigment. We extracted RNA using the Spectrum Plant Total RNA kit (Sigma), followed by an on-column DNase digestion to remove traces of genomic DNA. We then synthesized first-strand cDNA from 250 ng of total RNA using M-MLV, as described above. We compared expression levels from the different *I. horsfalliae* tissues with flower bud tissue from two other species: the cyanidin-producing *I. purpurea*, which represents the presumed ancestral condition, and the pelargonidin-producing, red-flowered *I. quamoclit*. The genetic basis of pelargonidin production in *I. quamoclit* has been the subject of intense study (Zufall & Rausher, 2004; Des Marais, 2008) and thus provides a useful measure of comparison.

Specific primers that amplified a single fragment of no more than 250 bp were designed in regions of the coding sequence that were identical for all three species. In addition

to the three candidate genes, we used the constitutively expressed translation elongation factor (*Ef1-a*) as a reference gene to control for differences in cDNA concentration. qPCRs were performed as described previously (Streisfeld & Rausher, 2009) on an ABI prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) with three replicates of each cDNA sample. All samples were first corrected for variation in PCR efficiency, according to Peirson *et al.* (2003). Relative quantification of *F3'b*, *Dfr* and *Ans* transcript abundance was analyzed as the logarithm of the mean fold-change in expression relative to *I. purpurea*, as described in Streisfeld & Rausher (2009).

Survey of mutations producing red flowers

In order to assess whether the type(s) of genetic change associated with the transition to red flowers in *I. quamoclit* and *I. horsfalliae* differs from expectation, we compiled reports in which spontaneous mutations and segregating variants in natural populations have been shown to be responsible for such color shifts. This comparison assumes that spontaneous mutations and segregating variants approximate the natural spectrum of mutations capable of producing a red-flowered phenotype. Our approach is similar to that used recently to determine whether fixed mutations among species that affect morphological characters differ qualitatively from the set of all mutations (Stern & Orgogozo, 2009). Reports were identified by searching the ISI Web of Science using a number of keyword combinations: 'flower anthocyanin', 'floral anthocyanin', 'flower color mutant' and 'anthocyanin mutant'. For each report, the gene in which the mutation occurred was tabulated. In addition, for each report, the nature of the mutation was noted. Mutations were categorized as 'functional' alterations if they eliminated enzyme functionality. By contrast, they were categorized as 'regulatory' if they altered expression levels of genes coding for anthocyanin pathway enzymes. Although this survey may not have identified all relevant reports, it should not be biased regarding the relative proportions of different types of mutation.

Results

Origins of red flowers

Parsimony reconstructions indicate that red-flowered lineages have arisen four times independently in the Astripomoeinae (in the lineages leading to *I. quamoclit*, *I. udeana*, *I. conzattii* and *I. horsfalliae*), and that there are no definitive cases of reversal to blue/purple flowers (200 of 200 trees sampled). However, parsimony analysis does not allow for the possibility that forward and reverse transitions could both occur along the same lineage. To address this possibility, we used stochastic mapping on multiple simulated realizations of 200 sampled trees (for the results of one realization, see Fig. 2). This approach indicates that transitions to red flowers occur more frequently than transitions away from red flowers [mean to red, 4.4 (0.8

SD); mean from red, 1.1 (1.4 SD)]. Moreover, in all cases, the transitions to red flowers in *I. quamoclit*, *I. udeana* and *I. horsfalliae* were evolutionarily independent (Fig. 2). Although some simulated realizations indicated a single common transition to red for *I. conzattii* and *I. horsfalliae*, we can reject this possibility because the two species have evolved the shared phenotype through different genetic mechanisms (*I. conzattii* does not produce pelargonidin derivatives in its flowers; Zufall, 2003). These results are robust to phylogenetic uncertainty and are consistent with previous reconstructions that did not include *I. horsfalliae* (Zufall, 2003).

Stem pigments

We used high-performance liquid chromatography (HPLC) to assay for the presence and identity of the colored anthocyanidins in stem extracts from *I. horsfalliae* and *I. udeana*. Although both species produce exclusively pelargonidin pigments in their flowers, they synthesize derivatives of cyanidins in their stems (Fig. 3). A similar result has also been obtained for *I. quamoclit* (Des Marais, 2008). *Ipomoea horsfalliae* makes a combination of cyanidin, peonidin (a modified cyanidin derivative) and traces of pelargonidin in its stems, whereas *I. udeana* produces only cyanidin in its stems. These results demonstrate that a functioning cyanidin branch of the anthocyanin pathway exists in these species and suggest, but do not prove, that changes in gene expression, rather than enzyme function, are responsible for the transition to pelargonidins in the flowers of these species.

Candidate genes

We next focused on the molecular genetic basis for the presence of pelargonidins in the flowers of *I. horsfalliae*. From *I. horsfalliae* floral cDNA, we PCR amplified and cloned full-length products of the three candidate genes that encode the anthocyanin enzymes responsible for the conversion of dihydroflavonols into the colored anthocyanidin pigments. All three genes showed sequence similarity to previously characterized anthocyanin pathway genes, and BLAST searches confirmed the homology of the *I. horsfalliae* sequences with anthocyanin genes from model species including *Ipomoea*. In addition, identical sequences were obtained when floral and stem tissues were used as cDNA templates, suggesting that we compared the same copy between the tissues in our expression analyses (see below). Finally, inspection of the sequences did not identify any obvious substitutions that introduced a frameshift or premature stop codon that would suggest nonfunctional enzymes.

Transgenic complementation

We used anthocyanin loss-of-function mutants from *A. thaliana* to test the function of three enzymes from *I. horsfalliae*. We independently introduced full-length coding sequences of

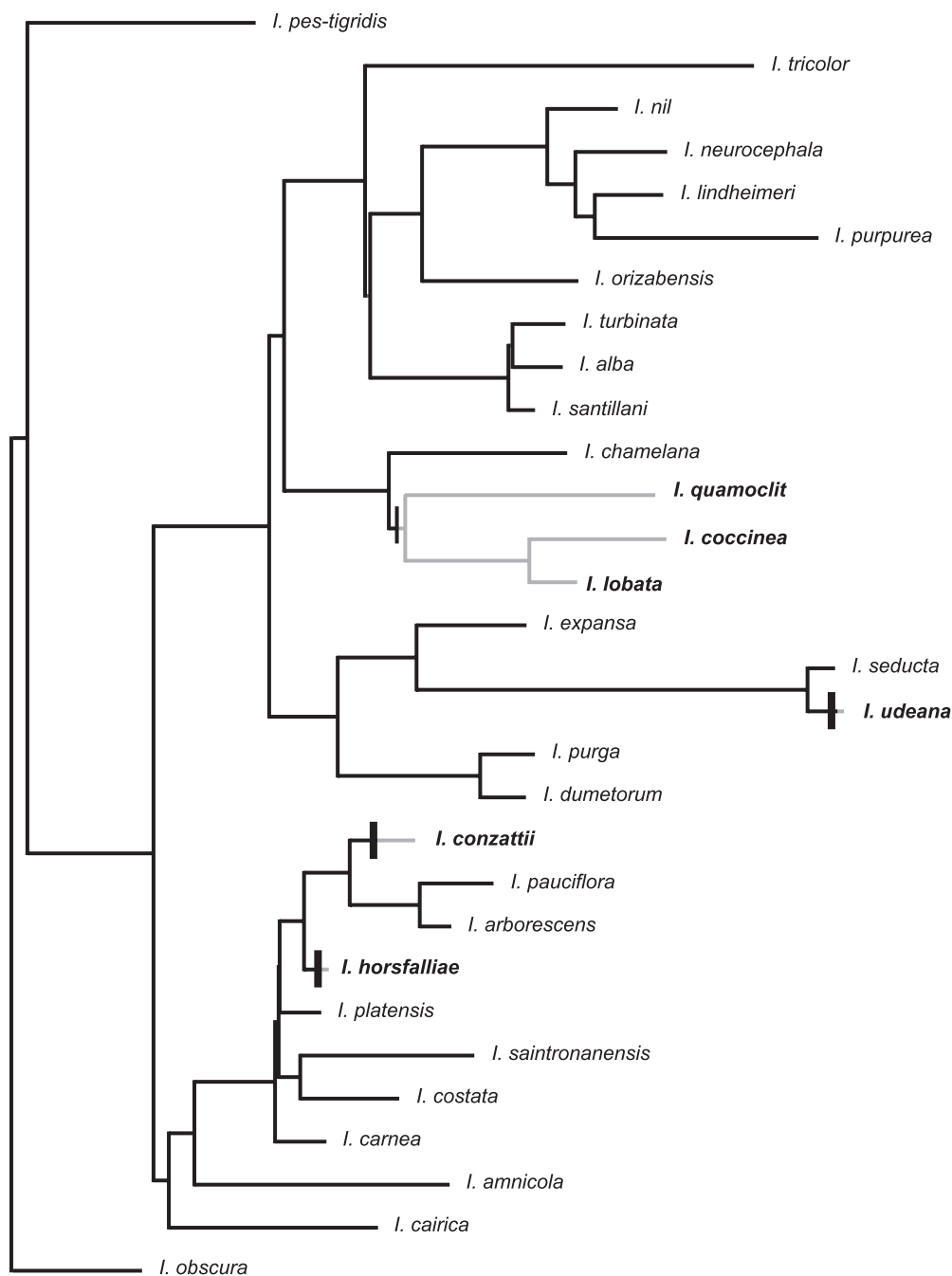


Fig. 2 Multiple independent origins of red flowers in the clade Astipomoeinae of the tribe Ipomoeae. Phylogenetic reconstructions of internal transcribed spacer (ITS) sequences from 30 taxa were performed with Bayesian estimation. One realization of the simulated character histories is shown from the stochastic mapping of character states. Character states are coded either as red (gray lines) or not red, which includes blue/purple, white and yellow flowers (black lines). The location of the transitions between states is denoted both by a change from black to gray along the branches and by black vertical bars. In this realization, four independent transitions occur to red flowers.

F3'h, *Dfr* and *Ans* from *I. horsfalliae* under the control of the CaMV 35S promoter into *A. thaliana* plants lacking activity of the enzymes encoded by these genes. For each construct, four independently transformed lines were able to complement the individual mutations in *A. thaliana* and restore anthocyanin production in the cotyledons of nutrient-stressed T2 seedlings:

anthocyanin production is induced in the cotyledons of these transgenic seedlings when grown under nutrient deprivation and high light (Fig. 4). Segregation of the transgene generated some seedlings that expressed anthocyanins and others that did not (Fig. 4). PCR of a gene-specific fragment demonstrated the presence of the transgene in those seedlings that produced

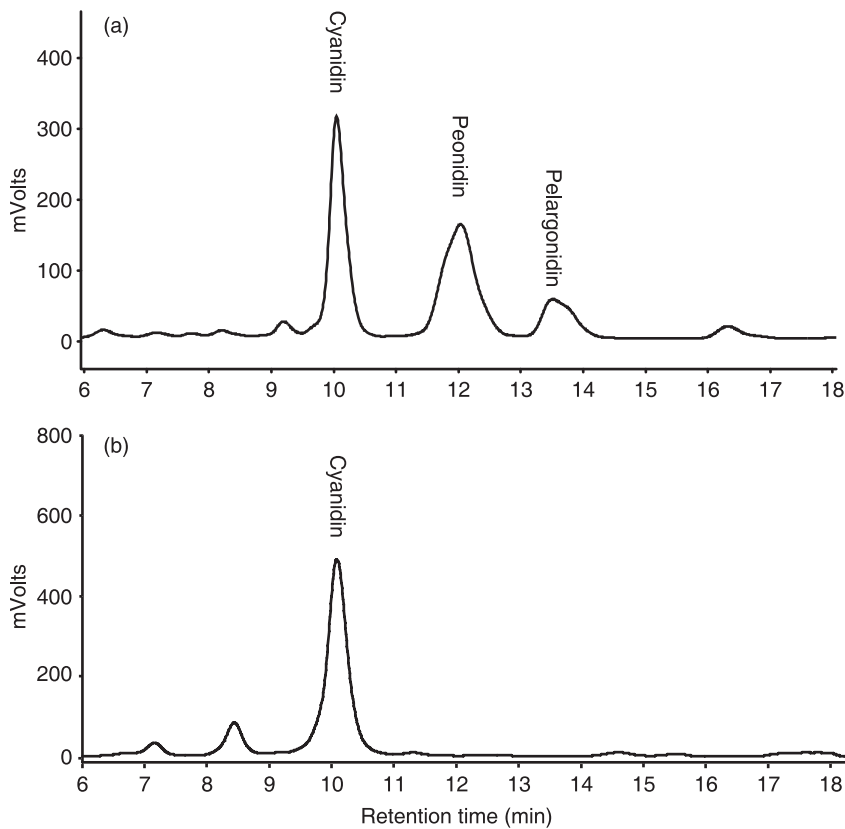


Fig. 3 High-performance liquid chromatograms of stem extracts of anthocyanidin pigments from (a) *Ipomoea horsfalliae* and (b) *I. udeana*. Peaks are labeled with their appropriate pigment, based on comparisons with standards of each of the six anthocyanidins.

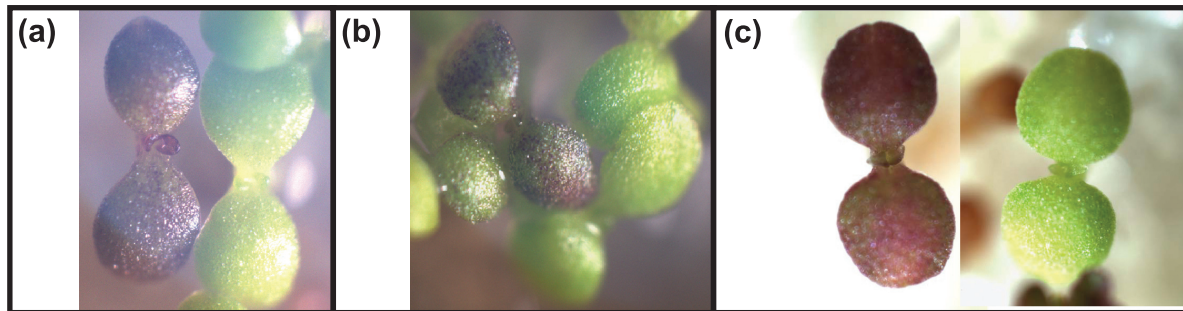


Fig. 4 Representative photographs demonstrating the transgenic complementation of *Arabidopsis thaliana* lines deficient in anthocyanin production using constructs containing full-length anthocyanin genes from *Ipomoea horsfalliae* driven by the constitutive cauliflower mosaic virus (CaMV) 35S promoter. (a) *tt7* *Arabidopsis* plants complemented with the *I. horsfalliae* *F3'h* gene; (b) *tt3* complemented with *Dfr*; (c) *tt18* complemented with *Ans*. Each set of photographs shows one seedling segregating for the presence of the transgene and complementing the mutation (dark pigment can be seen in the leaves) and another seedling from the same T2 generation lacking the transgene and failing to complement the mutation (no pigment is evident). Similar results were obtained among four independent lines transformed with each construct.

pigment (data not shown). These results demonstrate that the three candidate genes from *I. horsfalliae* encode enzymes that are functional *in vivo*. Because the CaMV 35S promoter overexpresses *F3'h* in transgenic *Arabidopsis*, we cannot rule out the possibility that the specific activity of this enzyme is reduced. However, the production of cyanidin in *I. horsfalliae* stem tissue (Fig. 3) indicates that *I. horsfalliae* *F3'H* retains substantial functionality.

Gene expression

The genes encoding the enzymes of the anthocyanin pathway typically are regulated at the level of transcription (Quattrocchio *et al.*, 2006a). We used qPCR to determine whether variation in transcript numbers of the three candidate genes could account for differential activities of the cyanidin and pelargonidin branches of the pathway in vegetative and floral tissues. Relative

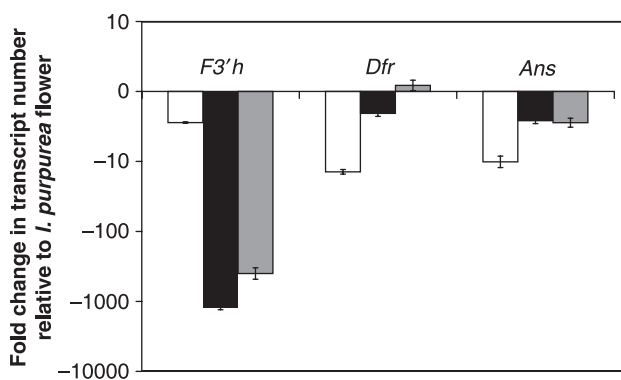


Fig. 5 Quantitative expression patterns of *F3'h*, *Dfr* and *Ans* from stem (white bars) and floral (black bars) tissue of *Ipomoea horsfalliae* relative to flower tissue of the purple-flowered *I. purpurea*. In addition, floral expression patterns from *I. quamoclit* (gray bars) are provided as a means for comparison. Expression is presented as the mean fold-change in transcript number for each sample relative to the purple-flowered *I. purpurea* (expression of *I. purpurea* is set to zero). Error bars represent ± 2 standard errors. Note the logarithmic scale.

to the cyanidin-producing flowers of *I. purpurea*, the flowers of *I. horsfalliae* produce, on average, 1200 fewer *F3'h* transcripts (Fig. 5). Likewise, flowers of *I. quamoclit* show a reduction in transcript number comparable with that of *I. horsfalliae* (see also Des Marais, 2008). Moreover, relative to *Dfr* and *Ans*, *F3'h* is markedly downregulated in the flowers of both of these species, compared with the flowers of *I. purpurea*. This result suggests that a lack of cyanidin production in *I. horsfalliae* flowers is caused primarily by a lack of *F3'h* expression.

By contrast, although *F3'h* transcript number in flowers of *I. horsfalliae* is greatly reduced relative to that in *I. purpurea* flowers, a similar downregulation in the stems of *I. horsfalliae* is not observed when relative transcript numbers are compared with the same *I. purpurea* floral sample (Fig. 5). Although we did not quantify the transcript number in the stems of *I. purpurea*, and thus cannot say whether there is a reduction in expression level of *F3'h* in *I. horsfalliae* stems relative to *I. purpurea* stems, our comparison of both tissue samples relative to a common standard indicates pronounced tissue-specific differences in the expression levels of *F3'h* in *I. horsfalliae*. Moreover, our evidence demonstrating the production of cyanidin in the stems of *I. horsfalliae* (Fig. 3) strongly suggests that any reduction in the observed expression levels of *F3'h*, *Dfr* and *Ans* in stems of *I. horsfalliae* relative to *I. purpurea* flowers probably has few functional consequences.

Survey of mutations

Our literature survey identified nine different mutations involving shifts between blue/purple flowers and red or pink flowers. Of the nine mutations, multiple types of mutagen were identified: six involved the insertion of a transposable

element, two involved frameshift mutations caused either by a single nucleotide insertion or deletion, and one was caused by a single point mutation that led to a premature stop codon (see Supporting Information Notes S1). Although we may have overlooked additional cases and the sample size of mutations is small, these examples appear to represent a largely unbiased estimate of the mutational spectrum for transitions from blue/purple to red flowers. In eight of these examples, red flowers were caused by the alteration of flux down different branches of the pathway (see Supporting information for details), whereas one eliminated the production of the flavonol copigments. Some of the eight examples involve the redirection of flux from the cyanidin branch to the pelargonidin branch, whereas others involve the redirection of flux from the delphinidin branch to the cyanidin branch. In the latter case, the delphinidin derivatives are blue/purple, whereas the cyanidin derivatives are red. In the eight cases involving the redirection of flux, the modification was achieved by 'functional' mutations that inactivated either the *F3'H* or *F3'5'H* enzyme. The elimination of flavonols in the ninth case was caused by inactivation of the flavonol synthase enzyme, which constitutes another example of a 'functional' mutation.

Although several of the spontaneous mutations involved the same gene (*F3'H*) as in the evolutionary transitions leading to *I. quamoclit* and *I. horsfalliae*, all nine cases involved 'functional' mutations and none consisted of 'regulatory' mutations. Although this may be a result of the relatively small number of reports examined, this observation suggests that, to produce a red or pink flower, 'functional' mutations occur more commonly than 'regulatory' mutations. It should be noted that, in none of these reports, were anthocyanins characterized in vegetative tissue. Consequently, we cannot be certain that these mutations abolished *F3'H* or *F3'5H* activity in vegetative tissue. Nevertheless, given the nature of the mutations, this inference seems reasonable.

Discussion

Parallelism in flower color change

In principle, parallel changes in flower color may occur at any or all of six different levels: (1) the phenotypic level; (2) the biochemical level; (3) the position in the anthocyanin pathway that alters flux (e.g. mutations downregulate or inactivate the same enzyme); (4) the type of mutation involved (functional or regulatory); (5) the type of regulatory mutation (*cis*- vs *trans*-); or (6) at the level of the individual nucleotide (mutations affect the same nucleotide). The four species *I. quamoclit*, *I. udeana*, *I. horsfalliae* and *I. conzattii* all evolved red, hummingbird-pollinated flowers independently. Thus, they exhibit parallelism at the phenotypic level. For three of these species (*I. quamoclit*, *I. udeana* and *I. horsfalliae*), this shift in hue is achieved through a common redirection of flux down the pelargonidin branch of the anthocyanin pathway, thus

demonstrating parallelism at the biochemical level. In *I. horsfalliae* and *I. quamoclit*, multiple lines of evidence suggest a parallel change in the pathway position at which the flux is controlled. In particular, the same gene, *F3'h*, appears to be downregulated in both cases. Moreover, in neither species does a mutation in DFR or ANS appear to contribute to the production of red flowers. Two lines of evidence presented here support this inference for *I. horsfalliae*: transgenic complementation of loss-of-function mutations in *Arabidopsis* indicates that both enzymes retain substantial activity on cyanidin precursors (Fig. 4); and the production of cyanidin derivatives in stems of *I. horsfalliae* indicates that DFR and ANS enzyme activities are sufficient to produce pigments *in vivo* (Fig. 3). Thus, flux down the cyanidin branch of the anthocyanin pathway appears to be blocked at the same position in both species.

The changes producing red flowers in these two species could also be considered to be parallel at the level of mutation type. In both cases, although the F3'H enzyme remains largely functional, transcript levels of *F3'h* are markedly reduced in flowers but not stems. Two lines of evidence presented here suggest that the enzymatic activity of F3'H is not substantially reduced in *I. horsfalliae*: (1) the enzyme complements a non-functional native copy of F3'H in *Arabidopsis* (Fig. 4); and (2) its activity is sufficient for the production of cyanidin derivatives in the stems of *I. horsfalliae* (Fig. 3). It thus appears that, in both species, the flower color shift is accomplished by a regulatory mutation affecting *F3'h* expression.

In *I. quamoclit*, this regulatory mutation appears to be largely a result of a *cis*-regulatory mutation in *F3'h* (Des Marais, 2008). One possible difference between the genetic changes in *I. quamoclit* and *I. horsfalliae* is that reduction in *F3'h* expression in *I. horsfalliae* may be achieved by downregulation of a transcription factor rather than by a *cis*-regulatory mutation in *F3'h*. Although we currently cannot rule out this possibility, which would require additional experiments to test *F3'h* promoter function in *I. horsfalliae* flowers, we believe it to be unlikely. Coordinated expression of blocks of anthocyanin pathway genes appears to be a common feature of the transcriptional regulation of the anthocyanin pathway (Dooner, 1983; Mol *et al.*, 1998; Koes *et al.*, 2005; Quattrocchio *et al.*, 2006a), although the set of loci coordinately controlled differs among species (Martin *et al.*, 1991). With the exception of *Ipomoea*, in all other eudicots that have been examined, transcriptional control is partitioned among early and late pathway genes, but the breakpoint varies among species (Mol *et al.*, 1998; Quattrocchio *et al.*, 2006a). However, in the two species of *Ipomoea* that have been extensively examined (*I. nil* and *I. purpurea*; and also in the monocot maize), all of the pathway genes, including *F3'h*, appear to be coregulated as a single block (Mol *et al.*, 1998; Tiffin *et al.*, 1998; Morita *et al.*, 2006). Provided that the pattern of coordinated regulation of anthocyanin pathway genes in *I. horsfalliae* is similar to that in *I. nil* and *I. purpurea*, if downregulation of *F3'h* in *I. horsfalliae* flowers is caused by alteration of a transcription factor, we would

expect to see marked downregulation of *Dfr* and *Ans* as well, but we do not (Fig. 5). Thus, the independent regulation of *F3'h* relative to *Dfr* and *Ans* suggests that changes in *cis*- to *F3'h* are responsible for the reduction of expression in flowers, potentially indicating that these species also exhibit parallelism at the level of the type of regulatory change. However, because we cannot eliminate the possibility that *F3'h* is regulated independently of the other anthocyanin genes by an unknown or duplicated copy of a transcription factor, this inference remains tentative.

Finally, because we have not identified the particular nucleotide change(s) responsible for the downregulation of *F3'h* in either species, we cannot determine whether there is parallelism at this level. Nevertheless, it is clear that the parallel phenotypic evolution of *I. quamoclit* and *I. horsfalliae* is conferred by parallelism at three, and perhaps four, lower levels of organization.

Our data are also suggestive that the shift to red flowers in *I. udeana* is conferred by lower level parallelisms. This transition is clearly parallel at the biochemical level, as flowers of this species produce only pelargonidin. In addition, although we have not identified the genetic change(s) responsible for the evolution of red flowers in *I. udeana*, it seems likely that this transition also involves regulatory mutations rather than functional mutations. Because *F3'h* and *Ans* are single-copy genes in *Ipomoea* (Hoshino *et al.*, 2001) and only one copy of the *Dfr* gene family retains activity on dihydroflavonols (Des Marais & Rausher, 2008), a functional mutation in any of these genes that blocks the cyanidin branch of the pathway in the flowers would also probably block cyanidin production in the stems. However, this scenario can be ruled out because *I. udeana* stems produce exclusively cyanidin (Fig. 3). The available evidence thus suggests that the shift to red flowers in *I. udeana* also involves parallel changes in the type of mutation involved (level 4 above), consistent with our observations for *I. quamoclit* and *I. horsfalliae*. However, until we are able to obtain floral tissue from *I. udeana* and further characterize the genetic changes associated with the shift to red flowers in this species, we will not know whether there is also parallelism at the position of flux blockage, in the type of regulatory mutation or at the nucleotide level.

Preferential targeting of mutations conferring red flowers

Our evidence suggests that there are multiple molecular routes that could contribute to the repeated evolution of pelargonidin-producing red flowers among species of *Ipomoea*. For example, in addition to data presented in this study, previously published results demonstrate that both functional and regulatory mutations in the gene encoding F3'H are capable of causing red flowers (Hoshino *et al.*, 2003; Zufall & Rausher, 2003; Des Marais 2008). In addition, although changes in the substrate specificity of DFR or ANS do not appear in either our survey

sample or among the *Ipomoea* species examined (suggesting that these mutations are probably rare), they nevertheless represent additional possible mechanisms for producing red flowers.

Although there are multiple ways to achieve this shift in flower color, the transitions from blue to red flowers in the three species of *Ipomoea* described here all appear to be a result of regulatory mutations involving the same gene. At least two possible scenarios can account for this: either regulatory mutations affecting *F3'h* expression are more common than other mutations, or natural selection fixes them preferentially. We can begin to evaluate these possibilities by examining data on the genetic basis of spontaneous and segregating mutations that confer red flowers. In a survey that included nine genetically characterized mutations, all of the mutations identified consisted of functional changes located in the coding regions of anthocyanin pathway enzymes, whereas none involved regulatory mutations (see Supporting Information Notes S1). Although it probably is dangerous to apply formal statistics to this limited data, we note that this difference in prevalence is statistically significant using Fisher's exact test ($P < 0.005$). Therefore, provided that this survey represents an unbiased estimate of the mutational spectrum for transitions from blue to red flowers, this result suggests that regulatory mutations arise less frequently than functional mutations. Although this pattern could be a result of a smaller mutational target size for regulatory mutations, our evidence from *Ipomoea* suggests that regulatory mutations are fixed repeatedly during the evolution of red flowers. Therefore, based on current evidence, our results suggest that regulatory mutations are being preferentially selected during these evolutionary transitions from blue/purple to red flowers in *Ipomoea*. We caution, however, that this inference is based on a limited sample of genetically characterized evolutionary transitions, and it may not be upheld when additional transitions are examined.

The potential role of deleterious pleiotropy

As described above, all of the genetically documented cases of evolutionary shifts between pigmented and unpigmented flowers involve alteration in the expression or activity of transcription factor proteins (Quattrocchio *et al.*, 1999; Schwinn *et al.*, 2006; Cooley, 2008; Streisfeld & Rausher, 2009). Moreover, in each of these cases, pigment production is absent in flowers but not in vegetative tissues. This tissue-specific difference in pigment production often is achieved via disruptions in duplicated copies of transcription factor proteins that reduce or eliminate their activity in floral tissue without affecting the expression of additional copies in other tissues (Quattrocchio *et al.*, 1993; Schwinn *et al.*, 2006). The common explanation for this pattern is that these mutations incur less deleterious pleiotropy compared with functional inactivation of pathway enzymes, because they eliminate the production of anthocyanins and other physiologically and ecologically important flavonoids only in

flowers (Rausher, 2006, 2008; Schwinn *et al.*, 2006; Whittall *et al.*, 2006; Streisfeld & Rausher, 2009).

Our observations that regulatory mutations seem to be involved preferentially in the transitions from blue/purple to red flowers in *Ipomoea* species raise the question of whether these regulatory mutations also experience minimal pleiotropy. Although we are unaware of any direct assessments of fitness associated with these various types of mutations, several pieces of indirect evidence suggest that the magnitude of pleiotropy may be reduced in mutations that affect the expression of these genes. First, in one survey of 110 angiosperm genera, Price & Sturgess (1938) found that 93% of species examined produced exclusively derivatives of cyanidin in leaves, whereas the remainder produced only delphinidin derivatives. The lack of pelargonidin derivatives in the foliage in this sample strongly suggests it is deleterious to produce these compounds in vegetative tissues, and therefore that the presence of an expressed and functional F3'H enzyme in these tissues is adaptive. This suggestion is supported by transgenic experiments in *Petunia* and *Arabidopsis*, in which it was found that an increase in the amount of the dihydroxylated flavonol, quercetin (which requires a functional F3'H; Fig. 1), provides significantly higher tolerance to UV-B radiation relative to the monohydroxylated kaempferol (Ryan *et al.*, 1998, 2001). Although not definitive, we believe that this evidence warrants that we continue to explore the hypothesis that regulatory mutations producing red flowers incur less deleterious pleiotropy than functional mutations causing the same phenotype.

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Supporting Information

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

Notes S1 Reported and molecularly characterized mutations known to change flower color from blue/purple to red.

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